AN INVESTIGATION INTO MICROBIAL BIOTRANSFORMATIONS OF ANTIMONY

Louise M. Smith

August 2001

A thesis submitted to De Montfort University in partial fulfilment of the requirements of the degree of Doctor of Philosophy

This thesis is entirely my own work and has at no time been submitted for another degree

I certify that this statement is correct

ACKNOWLEDGMENTS

The contribution of my *Mum* and *Dad* cannot be underestimated, without their love and support I would never have made it this far.

Thanks are due in no small measure to *Dr. Richard Jenkins*, not just for his help and advice throughout this project, but also for setting the scientific pace.

I would also like to thank the technical support teams at DMU. Particular mention must be made of *Mr Colin Boot*, *Mr Martin Edwards* and *Mr Tony Woodford*, who always found the time to search for an emergency gizmo or to magic life back into tired equipment.

Last, but not least, I would like to thank *Karsten*. Words are not enough.

Louise

ABSTRACT

Interactions of microorganisms, both prokaryotic and eukaryotic, with the metal antimony were studied. Of particular interest was the process of biomethylation.

Volatilisation of trimethylantimony from inorganic antimony substrate by mixed inoculum (of environmental source) enrichment cultures was demonstrated to occur. Trimethylantimony was the sole volatile antimony species detected in incubations designed to promote the growth of clostridia, no stibine or other volatile methylated species were detected. Two *Clostridium* sp. were isolated from environmental enrichment incubations and three characterised *Clostridium* sp. were demonstrated to possess a biomethylating capability. Up to 21 $\mu g.l^{-1}$ involatile methylantimony species were detected in the culture medium of monoseptic incubations of the characterised *Clostridium* sp. The relative quantities of involatile mono-, di- and trimethylantimony species produced during the course of the cultivation period is consistent with trimethylantimony oxide being a final product of antimony biomethylation, with mono- and dimethylantimony species appearing transiently in the cultures as intermediates of an antimony biomethylation pathway.

The fungi *Cryptococcus humicolus, Candida boidinii, Candida tropicalis*, *Geotrichum candidum* and *Saccharomyces cerevisiae* were all demonstrated to possess a similar antimony biomethylating capability. Volatile and involatile methylantimony species were detected, with involatile species being the predominant form. Both stibine and trimethylantimony were detected in culture headspace gases of fungal incubations. Levels of trimethylantimony were higher in incubations supplied with antimony III substrate, whilst stibine was the predominant volatile antimony species in incubations supplied with V valency substrate. *S.cerevisiae* demonstrated the highest stibine generating capability with up to 0.3% substrate being transformed. Regardless of substrate, overall antimony biomethylation efficiency (to both volatile and involatile species) was low, indicating that this biotransformation does not form the primary mode of resistance to the metal. Less than 0.1% of antimony III substrate was biomethylated by *C.humicolus*, the most productive species in terms of formation of methylantimony compounds. The intracellular accumulation of methylated antimony species further belies the theory that antimony biomethylation constitutes a resistance mechanism. Study of *C.humicolus* revealed the biomethylation process to be enzymatic and inducible by arsenic but not by antimony. This may indicate that the enzymes of the arsenic biomethylation pathway are the likely biocatalysts for the biomethylation of antimony. The low efficiency of antimony biomethylation indicates that this is most likely a fortuitous process.

A number of Gram-positive cocci isolated from soil and sediment were demonstrated to bioreduce antimonate to an unknown inorganic antimony III compound concurrently with lactate oxidation and biomass formation (as measured by protein). Up to 48% of the supplied antimonate was bioreduced. The demonstration of dissimilatory antimonate respiration adds this metal to the increasing list of known "unusual" electron acceptors such as uranium, arsenic, selenium, iron and manganese.

These studies reveal some of the microbial interactions of microorganisms with the metal antimony, demonstrating the potential that microorganisms have to contribute to the biogeochemical cycling of antimony through biotransformation processes.

1 General Introduction

1.1 Antimony species in the environment

The element antimony in Group 15 of the Periodic Table has an atomic mass 121.8 and an atomic number 51. It exists in two isotopic forms, namely ^{121}Sb and ^{123}Sb, which have natural isotopic proportions of 57.21 and 42.79 respectively.[1] Antimony is distributed diffusively in the earth's crust at concentrations of 0.2-0.5 $mg.kg^{-1}$ [2, 3]. It occurs mainly in nature as stibnite (Sb_2S_3), and as its decomposition product valentinite (Sb_2O_3), and is commonly associated with ores of copper, lead and silver. The element finds numerous uses in such diverse applications as (1) a catalyst in polyester and ammonia manufacture, (2) in light bulbs as Flu-powder in fluorescent lighting and the glass of standard light bulbs and (3) as an additive to alloys such as lead solder, pewter, battery plates and lead shot for ammunition, facilitating increases in hardness, mechanical strength, corrosion-resistance and conductivity. It is also used as a medicinal compound both in veterinary medicine and for the treatment of various tropical parasitic diseases. By far the greatest usage of antimony however, is as a fire-retardant for synthetics and textiles.[4] Table 1.1 compares emissions of arsenic and antimony to the atmosphere from natural and anthropogenic sources. It can be seen that for both elements anthropogenic input to the environment exceeds that from natural phenomena. Antimony is classified as a "grey-list" substance by the European Economic Community [5] and since 1979 has been one of the U.S. Environmental Protection Agency's 129 priority pollutants.[6]

Table 1.1 Comparison of global emissions ($kt.y^{-1}$) of antimony and arsenic to the atmosphere (source ref.)[7].

Human source	Antimony	Arsenic	Natural source	Antimony	Arsenic
Energy production [a]	1.3	2.2	Wind-borne dust [b]	0.8	2.6
Mining	0.1	0.1	Sea-salt spray	0.6	1.7
Smelting and refining	1.4	12.3	Volcanic activity	0.7	3.8
Manufacturing	-	2.0	Forest fires	0.2	0.2
Commercial	-	2.0	Biogenic sources	0.3	3.9
Waste incineration	0.7	0.3			
Total	3.5	18.9	Total	2.6	12.2

[a] Energy production includes coal, oil and gas. [b] Includes industrial sources of dust.

In contrast to other heavy metals, data regarding occurrence and speciation of antimony within environmental compartments is not extensive. Antimony concentrations in seawater have been reported to be about 0.2 $\mu g.l^{-1}$ mainly in the form of $Sb(OH)^-_6$, although $HSbO_2$, monomethylstibonic acid and dimethylstibinic acid have also been detected.[8-10] Methylantimony species have also been observed in plants such as pondweed, liverwort and mosses growing in locations influenced by drainage from mine workings.[11, 12] Data from exposure trials near an antimony smelter indicated that high levels of antimony observed in vegetation are due to atmospheric deposition as opposed to vegetative uptake.[13]

Within sediments, antimony has been shown to be associated with relatively immobile iron and aluminium compounds,[3] and that the leaching from such sediments is significant only upon a short-term basis. $Sb(OH)_6^-$ is thought to be the primary antimony species found in soils and sediments.[14] Mobility of antimony from sediments is dependant upon interactions between particulate matter and interstitial water. In addition to complexing with iron and aluminium, antimony also complexes with soil humates.[10] Redox conditions perform a primary role in the mobilisation of antimony since the status of particularly iron is strongly affected by redox conditions.[15] In addition, iron-reducing bacteria, which are thought to catalyse the majority of iron reduction in anoxic sediments,[16] may influence the degree of antimony mobilisation through dissolution of iron oxide.

Environmental processes consist of a complex interplay of biotic and abiotic processes; it is often difficult therefore to determine whether a metal species has arisen through chemical or biological means. Photochemical radiation for example, can induce the formation of metal-carbon bonds. Akagi and Takabatake [17] demonstrated that irradiation of mercuric sulphide solution in the presence of acetate ions resulted in the formation of methylmercury. Transmethylation reactions can occur though biotic or abiotic means. The abiotic transfer of methyl groups from methyltin to mercury to form methylmercury and from methyllead to tin forming methyltin have both been reported.[18] The methylation of lead and tin has also been demonstrated to occur by methyliodide, a biogenic compound.[19, 20] Under such circumstances, it becomes difficult to differentiate sharply between abiotic and biotic processes.

1.2 Microbial biotransformations of antimony compounds

The biochemical modification of the metals and metalloids mercury, tin, arsenic, antimony, selenium and tellurium through formation of volatile metal hydrides and methylated species (volatile and involatile) performs a fundamental role in determining the environmental processing of these elements. In general, the formation of such species increases the environmental mobility of the element, and can result in bioaccumulation in lipophilic environments. The toxicity of volatilised and methylated species is often higher than that of the corresponding inorganic metal compound due to their increased lipophilicity.[21, 22] Arsenic and selenium (and likely antimony) are exceptions to this rule however, possibly because of their respective abilities to take the place of phosphate and sulfate and a predilection for breaking cysteine bridges causing destruction of enzymatic function when in the inorganic form. It has been suggested that the formation of anionic methylarsenic compounds is a strategy for detoxification of the metal.[23-25] No comparative studies regarding the toxicity of organic and inorganic antimony compounds exists to date. However, it is generally conjectured that the toxicity of antimony compounds will closely resemble that of the related arseno species.[26-28] It is clear therefore that the toxicity of metals and metalloids is dependant upon their speciation, and that it is of primary importance to determine not only the amount of a metal within an environmental niche, but also to establish what forms the metal takes.

Whilst the microbial biotransformations of metals such as arsenic and mercury are well established, having been researched as in the case of arsenic from the late 19th century,[29-31] that of the metal antimony has received comparatively little attention until recent times. After the work of Barnard, Challenger and Ellis demonstrated that the fungus *Penicillium notatum* could volatilise inorganic antimony supplied to culture incubations,[32] it was not until the 1990's that interest in biotransformations of the element resurfaced. In 1990, Richardson [33] proposed that volatilisation of inorganic phosphorous, antimony and arsenic from PVC mattress covers by fungi was a possible primary cause of sudden infant death syndrome (SIDS).[34] The filamentous fungus *Scopulariopsis brevicaulis* was strongly implicated. A possible link between liver and serum concentrations of antimony and SIDS was also suggested and expounded in a television documentary in 1994.[35] The data supporting this theory were not published, but were been mentioned by Fleming *et al.* (1994).[36] A subsequent study refuted these

claims,[37] as did the findings of two U.K. Department of Health reports,[38, 39]. Recent reports [40, 41] however, support the existence of a link between elevated antimony levels in urine and serum and SIDS. A number of groups have since reported on the capability of *S.brevicaulis* to produce volatile antimony species from inorganic antimony substrate.[42-45] Whilst Gates [42] and Pearce [45] reported that no antimony volatilisation was performed by this fungus, Andrewes *et al.* [43] demonstrated the presence of picogram amounts of trimethylstibine in the headspace of *S.brevicaulis* cultures, a total of 0.9 ng trimethylstibine was produced in 1 month. This amount is far below the detection limits of the aforementioned reports (50 ng and 5 μg for Gates and Pearce respectively). The higher amounts of volatilised antimony (non-speciated) reported by Jenkins *et al.* [44] for similar aerobic trapping experiments with *S.brevicaulis* are likely a result of increased biomass amount arising from use of a richer incubation medium (malt extract vs. inorganic salts medium). This group facilitated speciation of the trimethylstibine produced by *S.brevicaulis* by utilising a biphasic, aerobic-anaerobic incubation regime. Anaerobic incubation conditions were implemented at the late log phase of growth (shown to be the most productive phase of trimethylantimony by the fungus). Although the likelihood of oxidation of the antimony species of interest was reduced by this method, its use did however raise questions as to the normal environmental processing of antimony by this organism. Whilst *S.brevicaulis* does not grow under anaerobic conditions, it cannot necessarily be assumed that the volatile antimony observed was formed only during the aerobic phase of incubation and preserved from oxidation during the later phase. It is possible that implementation of anaerobic incubation conditions affected the physiology and metabolism of the fungus such that stress products were formed, for example prior to sporulation. Both Andrewes *et al.* [43] and Jenkins *et al.*,[44] despite identification of the antimony volatilising capability of *S.brevicaulis*, reported problematic quantification and at times intermittent detection. The intermittent detection of trimethylstibine in aerobic incubations has been suggested to be due to the high susceptibility of this species to oxidation [46-48].

Andrewes *et al.* [43] also reported the detection of involatile dimethyl and trimethylstibine species in culture media of *S.brevicaulis* incubations. Dimethylstibine was the predominant involatile species in incubations supplied with antimony trioxide and in incubations supplied with high levels of potassium antimony tartrate (1000 $mg.l^{-1}$). Levels of di- and trimethylated antimony were observed to be roughly equivalent at

lower loadings of potassium antimony tartrate substrate (10 $mg.l^{-1}$). This group additionally demonstrated that the labelled methyl group of L-methionine-*methyl-d_3* is transferred intact to these species,[43] indicating that S-adenosylmethionine is the methyl donor involved in antimony biomethylation in this organism, as it is for arsenic biomethylation [49]. Antimony biomethylation by *Phaeolus schweinitzii* has also been recently demonstrated [50]. Antimony volatilisation by other fungi known to volatilise arsenic (*Penicillium* spp., *Aspergillus* spp.) has been reported not to occur.[51] Analysis of the potential for production of involatile antimony by these species has not been reported.

Detection of trimethylstibine in the headspace of undefined, mixed cultures, grown under anaerobic conditions has been reported previously [42, 46, 47]. The report of Gürleyük *et al.* [46] demonstrating antimony volatilisation from inorganic antimony substrate, supplied in either valency state, by soil bacteria enrichment cultures represents the first characterisation of a volatile antimony species from inorganic antimony since the work of Barnard in the 1940's[32]. Enrichment cultures were designed such that the growth of denitrifying bacteria was promoted. However, the specific identity of bacteria responsible for the biomethylation of antimony was not elucidated. Gates *et al.* [42] reported the presence of volatile trimethylstibine from potassium antimony tartrate in enrichment cultures of fermentative bacteria (in particular clostridia) using pond sediment as an inoculum source. Jenkins *et al.* [47] reported positive detection of volatile trimethylstibine in the headspace of cultures using soil and sediment from various habitats as inoculum and for three types of enrichment regime, namely enrichment for fermentative, denitrifying, and methanogenic bacteria. These reports demonstrate that as for arsenic, different metabolic groups of prokaryotic organisms are able to methylate antimony and that this capability is widely distributed in the terrestrial environment. To date, the only reports of biomethylation of inorganic antimony substrates by monoseptic bacterial cultures is that of Michalke *et al.* [52] who reported the biomethylation of inorganic antimony by three methanogenic archaea species, by *Clostridium collagenovorans* and by *Desulfovibrio vulgaris*. Volatile trimethylstibine was detected as the sole methylantimony species detected in culture headspace gases of these cultures. As yet, no analysis of the liquid phase of culture incubations for the presence of methylantimony species has been reported for bacterial cultures, either monoseptic, or of mixed inoculum. Since methylantimony species are highly susceptible to

oxidation, analysis of the liquid phase of culture incubations will likely increase the probability of detection, and may enable elucidation of the mechanism of antimony biomethylation. Species such as monomethylstibonic acid, dimethylstibinic acid and trimethylantimony oxide could be formed as intermediates, or as final biotransformation products, of a biomethylation pathway analogous to that established for biomethylation of arsenic, i.e. the Challenger mechanism.

As with biomethylation of antimony, the fungal reduction of inorganic antimony compounds has not been extensively demonstrated. Ultra-trace levels of stibine (SbH_3) were reported as being present in the headspace of *S.brevicaulis* cultures supplied with potassium antimony tartrate.[43] Currently, this represents the sole report of fungal bioreduction of antimony. Bacterial bioreduction of antimony is likewise under-represented in the literature. Michalke *et al.* [52] detected stibine in *Methanobacterium formicium* cultures supplied with antimony trichloride. The bioreduction of trimethyldibromoantimony to trimethylstibine by a monoculture of *Pseudomonas fluorescens* [46] has also been described. The reduction of arsenic V to the III valency form has been demonstrated for a number of microorganisms. Indeed this is the first step required for biomethylation from arsenic V substrates.[53-55] If the Challenger mechanism of arsenic biomethylation is assumed for the biomethylation of antimony, the reports of trimethylstibine production from potassium hexahydroxyantimonate (antimony V) [44, 46] are indicative of the reductive capability of certain microorganisms. Respiratory reduction (i.e. utilisation of energy derived from the reductive process for growth) has been described for the higher valences of arsenic and selenium,[56-58] as well as iron, uranium and manganese [16, 59]. As yet however, no reports of respiratory reduction of antimony exist despite the similarity in reduction potentials for arsenic V and antimony V.

The first reports of bacterial oxidation of arsenic were as early as 1918.[60-62] As with reduction of the metalloid, they appear to represent a detoxification mechanism. Bio-oxidation of antimony trioxide by the bacterium *Stibiobacter senarmontii* with coupling of the derived energy to biosynthesis has been reported.[63] Mention has also been made of the ability of *S.brevicaulis* to oxidise antimony III to antimony V;[43] supporting data was not provided. To date these are the only reports of microbial bio-oxidation of antimony.

1.3 Microbial resistance to antimony compounds

Microorganisms are able to employ a number of strategies to ensure resistance to metal ions. Wood and Wang [64] listed these as:

1. Energy-driven efflux pumps which keep levels of the toxic element low in the interior of the cell. e.g. cadmium, arsenic.
2. Enzymatic oxidation (e.g. arsenic III to arsenic V) or reduction (mercury II to elemental mercury) resulting in a less toxic form of the element.
3. Biosynthesis of intracellular polymers that trap metal ions from solution, e.g. cadmium, calcium, nickel, copper.
4. Binding of metal ions to metal cell surfaces, e.g. iron to siderophores.
5. Precipitation of insoluble metal complexes at cell surfaces, e.g. metal sulphides, metal oxides.
6. Biomethylation and transport through cell membranes by diffusion controlled processes.

It is worth noting that although included in this list, biomethylation of metals is not universally regarded as a resistance mechanism.[65]

Arsenate being of similar molecular dimensions to phosphate can enter cells via the inorganic phosphate transport (Pit) system [66] to be subsequently reduced by arsenate reductase and exported as arsenite by a highly specific oxy-anion efflux pump.[65, 67] Despite the apparent contradiction in transforming one molecular species (arsenic V) to a more toxic form (arsenic III), it is conjectured that the cell expends less metabolic energy performing the biotransformation than in attempting to distinguish between phosphate and arsenate at the uptake level.[68] Interestingly, certain microorganisms have developed increased resistance to arsenic by virtue of possessing an inducible phosphate transport system (Pst - phosphate specific transport) that has a higher selectivity for phosphate over arsenate.[69] Plasmid mediated resistance to antimony III compounds has been found in a wide variety of bacteria. It appears that the arsenate reductase - arsenite efflux couple is responsible for conferment of antimonite resistance.[69-71] Antimony III compounds are thought to enter the cell via passive diffusion since they are unionised at physiological pH.[72] To date, five *ars* operons have been identified and sequenced on

plasmids of S*taphylococcus sp.*, and plasmids and the chromosome of *Escherichia coli*.[69, 71, 73-75] These operons are structurally related, comprising: *ars*R, trans-acting regulatory protein stimulated by arsenate, arsenite, antimonite and bismuth; *ars*D, non-inducible trans-acting regulatory protein; *ars*A, ATP-ase sub-unit; *ars*B, efflux pump stimulated by arsenite; and *ars*C, arsenate reductase. *Ars* operons of *Staphylococcus* lack the *ars*A gene and use membrane potential to drive the arsenite efflux pump. The mechanism of prokaryotic resistance to antimony V compounds is unclear.[76, 77]

In contrast to the detailed knowledge of prokaryotic resistance to arsenic (and antimony), little is known about the mechanisms of resistance in yeast, fungi and algae. Kneer *et al.* [78] and Ecker *et al.* [79] demonstrated that yeast and fungi are able to withstand high metal concentrations through chelation with phytochelatin peptides, binding to proteins (e.g. cadmium and metallothionen) and the use of multi-drug resistance (*mdr*) transporters. Cervantes *et al.* (1986) [65] comment however that neither of these mechanisms is responsible for arsenic resistance. There have been recent reports in the literature of the isolation of inducible genes (ACR1, ACR2, ACR3), which are similar in structure to *ars* genes of *Staphylococcus* and appear to confer resistance to arsenate, but not for arsenite.[80] The similarities in physico-chemical and toxicological properties of arsenic and antimony have been much commented on. [81, 82]. Despite this, much remains to be elucidated regarding microbial resistance to antimony.

1.4 Speciation of antimony compounds

Speciation is a term that has been subject to varied interpretation.[83] It is used here in this thesis, in agreement with current usage, to describe the precise identification of a chemical compound. Since the development of hyphenated instrumental analytical techniques with increased sensitivity and selectivity, analysis and speciation of organometallic compounds in environmental samples has become a continuously developing field. The conventional method for antimony speciation has been gas chromatography-atomic absorption spectrometry (GC-AAS), although other spectroscopic techniques such as atomic fluorescence spectroscopy (AFS) or inductively coupled plasma-atomic emission spectroscopy (ICP-AES) have been used. To enable analysis of involatile species present in solid or liquid phases, GC-AAS is preceded by a derivatisation step enabling volatilisation to the gaseous phase. For the hydride forming elements, arsenic, antimony, bismuth, germanium, selenium, tellurium

and tin, it is possible to use a chemical agent such as sodium borohydride for the derivatisation step. This technique of hydride generation enables improvement of detection limits by eliminating the presence of non-hydride generatable compounds from the analyte stream, that could provide spectral line or vaporisation interferences were non-chemical derivatisation means to be used. Once analytes are in the gaseous phase, separation by gas chromatography can be simply applied. The major disadvantage of hydride generation, however, is that true speciation cannot be performed; only information about the oxidation state of the element or degree of alkylation of an alkylated compound can be determined. Hence, trimethylantimony dichloride and trimethylantimony oxide will both upon derivatisation form a trimethylstibine peak. It should also be considered that some species are not hydride generatable under normal conditions, e.g. arsenobetaine, arsenocholine [84] and arsenolipids.[85] A combination of physico-chemical techniques must be employed to achieve speciation, e.g. microwave digestion or UV-photolysis preceding hydride generation. A further problem, particularly with antimony speciation, is that hydride generation tends to produce more than one product from a single analyte, e.g. hydride generation of a trimethylated antimony compound can, in addition to trimethylstibine, result in the formation of stibine, monomethylstibine, and dimethylstibine.[86, 87] This phenomenon is known as demethylation or dismutation and can be limited, although not necessarily eliminated, by manipulation of hydride generation reaction conditions.

A number of interferences have been reported for atomic absorption. Background absorption, i.e. the absorption of radiation by metal oxides, hydrogen molecules and OH radicals, can usually be remediated through the use of a blank solution. Likewise, spectral line interferences are generally not a significant problem due to the relatively narrow bandpass of the AAS monochromator. Vaporisation and ionisation interferences can however, impact seriously upon AAS analyses. Vaporisation interferences arise from chemical reactions within the sample matrix that affect the rate of release of analyte ions. To prevent complexing of calcium with phosphate during calcium analysis for example, low levels of strontium or lanthanum can be added to the sample. These preferentially bind with phosphate, releasing calcium for analysis. The use of hotter flames, such as acetylene/nitrous oxide (as opposed to acetylene/air) also tends to reduce these interferences by virtue of increased thermal decomposition within the flame. Ionisation interferences occur at elevated flame and furnace temperatures, which

cause atoms with low ionisation potentials to be ionised. This results in a lowered sensitivity, since the population of ground state atoms and neutral free atoms in excited state is reduced. Reduction of flame and furnace operating temperatures or addition of a more easily ionisable element as suppressant, such as potassium, caesium or strontium, can alleviate this interference. This will however, have a detrimental effect upon vaporisation interference.

For true speciation of involatile organometallic compounds, techniques such as HPLC-separation or voltammetry must be employed. HPLC separation of antimony species is currently problematic due to a lack of antimony standards. To date, separations have been described for (1) potassium antimony tartrate, potassium hexahydroxyantimonate and trimethylantimony oxide using strong ion exchange with phthalic acid or 4-hydroxybenzoic acid as eluent,[88] (2) potassium hexahydroxyantimonate and trimethylantimony (oxide or chloride) using anionic exchange with potassium hydroxide as eluent [89] and (3) potassium antimony tartrate and potassium hexahydroxyantimonate using anionic exchange with a complexing mobile phase (ethylene diaminetetra-acetic acid, EDTA),[89] or anionic exchange with phthalic acid as eluent.[90] The only current liquid chromatography method to achieve separation of both III and V valency inorganic species, and an organoantimony species [88] noted that antimony III was strongly retained on the column, and that problems were incurred attaining elution of antimony III whilst maintaining resolution of the antimony V and organoantimony species. The use of gradient elution was not suitable since this would result in different matrix composition for the analyte species. As yet, the only HPLC separation technique that has been described for the separation of organoantimony species used anionic separation to achieve separation of antimony V complexes with α-hydroxy acids;[91] analysis of antimony III complexes was not made. The paucity of literature regarding analysis of involatile antimony species is most likely due to a lack of research interest in antimony enviro-chemistry until recent times.

Inductively coupled plasma-mass spectrometry (ICP-MS) has become in recent times the detector of choice for trace metal analyses. The major advantage of ICP-MS over other techniques is the possibility of simultaneous multi-element analysis, in addition to low detection limits for most elements. Although the use of ICP-MS has been limited in the past due to the relatively high purchase and running costs when compared to other

analytical techniques, these costs have fallen in recent years. Sample introduction methods include nebulisation, hydride generation and electrothermal vaporisation. Interferences can be spectroscopic and/or non-spectroscopic. Interferences caused by the sample matrix comprise the majority of the latter and can, in the main, be removed by sample dilution or by removal of the offending species from the analyte mixture. Spectroscopic interferences are subdivided: (1) isobaric interferences – two elements within the sample having isotopes with substantially the same mass, i.e. that differ by less than one unit; (2) polyatomic ion interference – interactions between species in the sample matrix, atmosphere or plasma can interact leading to the formation of molecular species that overlap the analyte of interest; (3) oxide and hydroxide interference – interaction of the analyte metal with components of the matrix, solvent or plasma gases resulting in the formation of MOH^+ or MO^+ species that can potentially overlap the peak of one of the analyte ions. In general, spectroscopic interferences can be overcome by use of a blank or selection of an alternative, and unfortunately usually less sensitive, isotope for analysis. Reduction of oxide interference can be achieved by modification of operating conditions such as plasma gas composition, oxygen elimination and radio frequency power. Despite these limitations, ICP-MS has been applied successfully to the analysis of standard antimony species [87, 92] and antimony species in environmental samples.[93-96]

1.5 Scope of this Study

The aim of this work was to investigate the extent to which microorganisms, both bacteria and fungi, interact with the heavy metal antimony. This included work to extend the range of characterised microorganisms capable of biomethylating antimony and identification of environmental sources of microorganisms capable of biotransforming inorganic forms of the element. Environmental samples from a variety of habitats were screened for a biomethylating capability, and enrichment culture conditions that can lead to antimony biomethylation were investigated. It was of interest to attempt the isolation and characterisation of microorganisms capable of antimony biomethylation from environmental sources.

The fungus *Cryptococcus humicolus* was studied in detail with regard to characterising the biomethylation of antimony more fully. A number of different antimony substrates were tested as biotransformation substrates, and parameters such as substrate type and

concentration, and time of presentation to culture incubations were investigated. Uptake mechanisms of antimony and arsenic were also studied. This organism is a known biomethylator of arsenic; it was of interest therefore, to perform comparative studies and to investigate the effect of arsenic upon antimony biomethylation by this organism.

Analysis of both volatile and involatile antimony species in culture incubations was performed throughout. This required use, and at times development, of coupled analytical techniques such as solid-phase-micro-extraction-GC-MS, gas chromatography-atomic absorption spectroscopy and HPLC-atomic fluorescence spectroscopy.

For the sake of readability, the strict application of the term metalloid to describe the element arsenic has been avoided in this thesis, and both antimony and arsenic are described as `metal' throughout. To avoid confusion, current microbial nomenclature is used when discussing retrospective publications.

References for Chapter 1

1. IUPAC, Comission on atomic weights and isotopic abundances. International Union of Pure and Applied Chemistry, (1989).
2. IRPTC, Antimony Series: Scientific reviews of Soviet literature on toxicity and hazard of chemicals. Centre of International Projects, GNKT, Moscow, (1984).
3. J. M. Brannon and W. H. Patrick Jnr., Fixation and mobilization of antimony in sediments. *Environmental Pollution (Series B)* **9**, 107 (1985).
4. W. Sloof, P. F. H. Bont, J. M. Hesse, and B. Loos, Antimony and antimony compounds. RIVM (National Institute of Public Health and Environmental Protection), (1992).
5. EEC, On the evaluation of the control of risks of existing substances. European Economic Community, Brussels, (1993).
6. U.S. EPA, Water related environmental fate of 129 priority pollutants. Volume 1: Introduction and technical background, metals and inorganics, pesticides and PCB's. U.S. Environmental Protection Agency, Washington, (1979).
7. L. B. Clarke and L. L. Sloss, Trace elements. *IEA Coal Research* **49**, 15 (1992).
8. B. A. Fowler and P. L. Goering, *in* Metals and their compounds in the environment: Occurrence, analysis and biological relevance E. Merian, Vol. 743. 1991.
9. M. O. Andreae and P. N. Froelich, Arsenic, antimony, and germanium biogeochemistry in the Baltic Sea. *Tellus* **36B**, 101 (1984).
10. M. O. Andreae, J.-F. Asmode, P. Foster, and L. Van't dack, Determination of antimony(III), antimony (V), and methylantimony species in natural waters by atomic absorption spectrometry with hydride generation. *Analytical Chemistry* **53**, 1766 (1981).
11. P. J. Craig, S. N. Forster, R. O. Jenkins, and D. M. Miller, An analytical method for the detection of methylantimony species in environmental matrices: methylantimony levels in some UK plant material. *Analyst* **124**, 1243 (1999).
12. M. Dodd, S. A. Pergantis, W. R. Cullen, H. Li, G. K. Eigendorf, and K. J. Reimer, Antimony speciation in freshwater plant extracts by using hydride generation-gas chromatography-mass spectrometry. *Analyst* **121**, 223 (1996).
13. N. Ainsworth, J. A. Cooke, and M. S. Johnson, *in S.* Maeda, *in* The chemistry of organic arsenic, antimony and bismuth compounds S. Patai, Vol. 725. John Wiley & Sons Ltd., London, 1994.
14. A. M. Ure and M. L. Berrow, "The elemental constituent of the soils," p. p155. Royal Society of Chemistry, London, 1982.
15. H. W. Schmitt and H. Sticher, *in* Metals and their compounds in the environment: Occurrence, analysis and biological relevance E. Merian, Vol. 311. 1991.
16. D. R. Lovely, E. J. Phillips, and D. J. Lonergan, Dissimilatory (Fe)III and Mn(IV) reduction. *Microbial Reviews* **55**, 263 (1991).
17. H. Akagi and E. Takabatke, Photochemical formation of methylmercuric compounds from mercuric acetate. *Chemosphere* **3**, 131 (1973).

18. Y. K. Chau, Occurrence and speciation of organometallic compounds in freshwater systems. *The Science of the Total Environment* **49**, 305 (1986).

19. A. W. P. Jarvie and A. D. Whitmore, Methylation of elemental lead and lead (II) salts in aqueous solutions. *Environmental Technology Letters* **2**, 197 (1981).

20. P. J. Craig and S. Rapsomanikis, Methylation of tin and lead in the environment - oxidative methyl transfer as a model for environmental reactions. *Environmental Science and Technology* **19**, 726 (1985).

21. J. S. Thayer, "Organometallic compounds and living organisms," p. Academic Press Inc., Orlando, Florida, 1984.

22. J. S. Thayer, "Environmental chemistry of the heavy elements: hydrido and organo compounds," p. VCH Publishers Inc., Weiheim, Germany, 1995.

23. O. S. Fatoki, Biomethylation in the natural environment: A review. *South African Journal of Science* **93**, 366 (1997).

24. K. D. Huysmans and W. T. Frankenberger Jnr., Arsenic resistant microorganisms isolated from agricultural drainage water and evaporation pond sediments. *Water, Air, and Soil Pollution* **53**, 159 (1990).

25. M. Shariatpanahi, A. C. Anderson, and A. A. Abdelghani, *in* Trace substances in environmental health D. D. E. *in* Hemphill, Vol. Part X, p. 383. University of MIssouri, Columbia, 1981.

26. L. Friberg, G. F. Nordberg, and V. B. Vouk, Handbook on the toxicology of metals. Volume II: Specific metals. *Elsevier Science Publisher B.V.* 27 (1986).

27. A. Leonard and G. B. Gerber, Mutagenicity, carcinogenicity and teratogenicity of antimony compounds. *Mutation Research* **366**, 1 (1996).

28. K. A. Winship, Toxicity of antimony and its compounds. *Adverse Drug Reaction and Acute Poisoning Reviews* **2**, 67 (1987).

29. B. Gosio, Azine di alcune muffe sui composti fissi d`arsenico. *Rivisto d`igiene e sanitá pubblica* **3**, 201 (1892).

30. B. Gosio, Zur Frage, wodurch die Gifitgkeit arsenhaltiger Tapeten bedingt wird. *Berichte der Deutschen Chemischen Gesellschaft* **30**, 1024 (1897).

31. C. R. Sanger, On the formation of volatile compounds of arsenical wall papers. *Proceedings of the American Academy of Arts and Sciences* **21**, 112 (1894).

32. D. Barnard, The attempted methylation of compounds of antimony, mercury, tin and lead by *Scopulariopsis brevicaulis* and *Penicillium notatum*. 9. University of Leeds, U.K., Leeds, (1947).

33. B. A. Richardson, Cot mattress biodeterioration and SIDS. *Lancet* **335**, 670 (1990).

34. B. A. Richardson, Sudden Infant Death Syndrome: a possible primary cause. *Journal of the Forensic Science Society* **34**, 199 (1994).

35. Cook Report, Cot death (poisonings). Central Television, UK., 1994).

36. P. J. Fleming, M. Cooke, S. M. Chantler, and J. Golding, Fire retardants, biocides, plasticiser, and sudden infant death. *British Medical Journal* **309**, 1594 (1994).

37. A. G. Howatson, W. J. A. Patrick, G. S. Fell, T. D. B. Lyon, and A. A. M. Gibson, Cot mattresses and sudden infant death syndrome. *Lancet* **345**, 1044 (1995).

38. P. Turner, Sudden infant death syndrome (SIDS). Department of Health, London, (1991).

39. S. Limerick, Expert Group to investigate cot death theories: toxic gas hypothesis. Department of Health, London, (1998).

40. C. E. Delves, C. E. Sieniawska, G. S. Fell, T. D. B. Lyon, C. Dezateux, A. Cullen, S. Variend, J. R. Bonham, and S. M. Chantler, Determination of antimony in urine, blood and serum in liver and lung tissues of infants by inductively coupled plasma mass spectrometry. *Analyst* **122**, 1323 (1997).

41. C. Dezateux, H. T. Delves, J. Stocks, A. Wade, L. Pilgrim, and K. Costeloe, Urinary antimony in infancy. *Archives of Disease in Childhood* **76**, 432 (1997).

42. P. N. Gates, H. A. Harrop, J. B. Pridham, and B. Smethurst, Can microorganisms convert methylated antimony trioxide or potassium antimonyl tartrate to methylated stibines? *Science of the Total Environment* **205**, 215 (1997).

43. P. Andrewes, W. R. Cullen, J. Feldmann, I. Koch, P. E., and K. J. Reimer, The production of methylated organoantimony compounds by *Scopulariopsis brevicaulis*. *Applied Organometallic Chemistry* **12**, 827 (1998).

44. R. O. Jenkins, P. J. Craig, W. Goessler, D. Miller, N. Ostah, and K. J. Irgolic, Biomethylation of inorganic antimony compounds by an aerobic fungus: *Scopulariopsis brevicaulis*. *Environmental Science and Technology* **32**, 882 (1998).

45. R. B. Pearce, M. E. Callow, and L. E. Macaskie, Fungal volatilisation of arsenic and antimony and the sudden infant death syndrome. *FEMS Microbiology Letters* **158**, 261 (1998).

46. H. Gürleyük, V. Van Fleet-Stalder, and T. G. Chasteen, Confirmation of the biomethylation of antimony compounds. *Applied Organometallic Chemistry* **11**, 471 (1997).

47. R. O. Jenkins, P. J. Craig, D. P. Miller, L. C. A. M. Stoop, N. Ostah, and T.-A. Morris, Antimony biomethylation by mixed cultures of micro-organisms under anaerobic conditions. *Applied Organometallic Chemistry* **12**, 449 (1998).

48. G. E. Parris and F. E. Brinckman, Reactions which relate to environmental mobility of arsenic and antimony. II. Oxidation of trimethylarsine and trimethylstibine. *Environmental Science & Technology* **10**, 1128 (1976).

49. W. R. Cullen, H. Li, S. A. Pergantis, G. K. Eigendorf, and A. A. Mosi, Arsenic biomethylation by the microorganism *Apiotrichum humicola* in the presence of L-methionine-*methyl-*d_3. *Applied Organometallic Chemistry* **9**, 507 (1995).

50. P. Andrewes, W. R. Cullen, E. Polishchuk, and K. J. Reimer, Antimony biomethylation by the wood rotting fungus *Phaeolus schweinitzii*. *Applied Organometallic Chemistry* **15**, 473 (2001).

51. R. O. Jenkins, P. J. Craig, W. Goessler, and K. J. Irgolic, Biovolatilization of antimony and sudden infant death syndrome (SIDS). *Human & Experimental Toxicology* **17**, 231 (1998).

52. K. Michalke, E. B. Wickenheiser, M. Mehring, A. V. Hirner, and R. Hensel, Production of volatile derivatives of metal(loid)s by microflora involved in anaerobic digestion of sewage sludge. *Applied and Environmental Microbiology* **66**, 2791 (2000).

53. F. V. Vidal and V. M. V. Vidal, Arsenic metabolism in marine bacteria and yeast. *Marine Biology* **60**, 1 (1980).

54. W. R. Cullen, H. Li, G. Hewitt, and K. J. Reimer, Identification of extracellular arsenical metabolites in the growth medium of the microorganisms *Apiotrichum humicola* and *Scopulariopsis brevicaulis*. *Applied Organometallic Chemistry* **8**, 303 (1994).

55. B. C. McBride and R. S. Wolfe, Biosynthesis of dimethylarsine by Methanobacterium. *Biochemistry* **10**, 4312 (1971).

56. D. K. Newman, E. K. Kennedy, J. D. Coates, D. Ahmann, D. J. Ellis, D. R. Lovley, and F. M. M. Morel, Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. *Archives of Microbiology* **168**, 380 (1997).

57. A. M. Laverman, J. S. Blum, J. K. Schaefer, E. J. P. Phillips, D. R. Lovley, and R. S. Oremland, Growth of Strain SES-3 with arsenate and other diverse electron acceptors. *Applied and Environmental Microbiology* **61**, 3556 (1995).

58. R. S. Oremland, J. S. Blum, C. W. Culbertson, P. T. Visscher, L. G. Miller, P. Dowdle, and F. Strohmaier, Isolation, growth, and metabolism of an obligately anaerobic, selenate-respiring bacterium, Strain SES-3. *Applied and Environmental Microbiology* **60**, 3011 (1994).

59. K. H. Nealson and D. A. Saffarini, Iron and manganese in anaerobic respiration: environmental significance, physiology and regulation. *Annual Review of Microbiology* **48**, 311 (1994).

60. H. H. Green, Description of a bacterium which oxidises arsenite to arsenate, and one which reduces arsenate to arsenite, isolated from a cattle dipping tank. *South African Journal of Science* **14**, 456 (1918).

61. J. M. Quastel and P. G. Scholefield, Arsenite oxidation in soil. *Soil Science* **75**, 279 (1953).

62. F. H. Osborne and H. L. Ehrlich, Oxidation of arsenite by soil isolate of *Alcaligenes*. *Journal of Applied Bacteriology* **41**, 295 (1976).

63. N. N. Lyalikova, Oxidation of trivalent antimony up to the highest oxides as a source of energy for the development of the new autotrophic organism Stibiobacter Gen. N. *Mikrobiologika* **205**, 1228 (1972).

64. J. M. Wood and H. K. Wang, *in* Chemical processes in lakes W. Stumm, Vol. 81. Wiley, 1985.

65. C. Cervantes, J. Guangyong, J. L. Ramirez, and S. Silver, Resistance to arsenic compunds in microorganisms. *FEMS Microbiology Reviews* **15**, 355 (1994).

66. G. R. Willsky and M. H. Malamy, Characterisation of two genetically seperable inorganic phosphate transport systems in *Escherichia coli*. *Journal of Bacteriology* **144**, 356 (1980).

67. P. Kaur and B. P. Rosen, Plasmid encoded resistance to arsenic and antimony. *Plasmid* **27**, 29 (1992).

68. G. Ji and S. Silver, Bacterial resistance mechanisms for heavy metals of environmental concern. *Journal of Industrial Microbiology* **14**, 1995).

69. S. Silver, K. Budd, K. M. Leahy, W. V. Shaw, D. Hammond, R. P. Novick, G. R. Willsky, M. H. Malamy, and H. Rosenberg, Inducible plasmid-determined resistance to arsenate, arsenite and antimony (III) in *Escherichia coli* and *Staphylococcus aureus*. *Journal of Bacteriology* **146**, 983 (1981).

70. B. P. Rosen, U. Weigel, C. Karkaria, and P. Gangola, Molecular characterisation of an anion pump. *The Journal of Biological Chemistry* **263**, 3067 (1988).

71. R. Rosenstein, A. Peschel, B. Wieland, and F. Goetz, Expression and regulation of the antimonite, arsenite, and arsenate resistance operon of *Staphylococcus xylosus* plasmid pSX267. *Journal of Bacteriology* **174**, 3676 (1992).

72. T. Gebel, S. Christensen, and H. Dunkelberg, Comparative and environmental genotoxicity of antimony and arsenic. *Anticancer Research* **17**, 2603 (1997).

73. D. B. Rajini Rani and A. Mahadevan, Plasmid mediated metal and antibiotic resistance in marine *Pseudomonas*. *Biometals* **5**, 73 (1992).

74. C. Cervantes and J. Chavez, Plasmid-determined resistance to arsenic and antimony in *Pseudomonas aeruginosa*. *Antonie van Leeuwenhoek* **61**, 333 (1992).

75. R. P. Novick and C. Roth, Plasmid linked resistance to inorganic salts in *Staphylococcus aureus*. *Journal of Bacteriology* **95**, 1335 (1968).

76. B. P. Rosen, Bacterial resistance to heavy metals and metalloids. *Journal of Biological Inorganic Chemistry* **1**, 273 (1996).

77. S. Silver, Genes for all metals - a bacterial view of the Periodic Table. *Journal of Industrial Microbiology and Biotechnology* **20**, 1 (1998).

78. R. Kneer, T. M. Kutchan, A. Hochberger, and M. H. Zenk, *Saccharomyces cerevisiae* and *Neuspora crassa* contain heavy metal sequestering phtyochelatin. *Archives of Microbiology* **157**, 305 (1992).

79. D. J. Ecker, T. R. Butt, E. J. Stenberg, M. P. Neeper, C. Debouk, J. A. Gorman, and S. T. Crooke, Yeast metallothionin function in metal detoxification. *Journal of Biological Chemistry* **261**, 16895 (1986).

80. P. Bobrowicz, R. Wysocki, R. Owsianik, A. Goffeau, and S. Ulaszewski, Isolation of three contiguous genes, ACR1, ACR2 and ACR3, involved in resistance to arsenic compounds in the yeast *Saccharomyces cerevisiae*. *Yeast* **13**, 819 (1997).

81. L. Friberg and C. G. Elinder, Biological monitoring of toxic metals. *Scandinavian Journal of Work and Environmental Health* **19**, 7 (1993).

82. A. Leonard, *in* Metals and their compounds in the environment: Occurrence, analysis and biological relevance E. Merian, Vol. 751. 1991.

83. B. Raspor, *in* Metals and their compounds in the environment: Occurrence, analysis and biological relevance E. Merian, Vol. 234. 1991.

84. X.-C. Le, W. R. Cullen, and K. J. Reimer, Decomposition of organoarsenic compounds by using a microwave oven and subsequent determination by flow injection-hydride generation-atomic absorption spectrometry. *Applied Organometallic Chemistry* **6**, 161 (1992).

85. J. J. Wrench and R. F. Addison, Reduction, methylation and Incorporation of arsenic into lipids by the marine phytoplankton *Dunaliella tertiolecta*. *Canadian Journal of Fish and Aquatic Science* **38**, 518 (1981).

86. M. Dodd, S. L. Grundy, K. J. Reimer, and W. R. Cullen, "Methylated antimony compounds-synthesis, hydride generation properties and implications for aquatic speciation," p. 207. 1992.

87. I. Koch, J. Feldmann, J. Lintschinger, S. V. Serves, W. R. Cullen, and K. J. Reimer, Demethylation of trimethylantimony in aqueous solution during analysis by using hydride

generation/gas-chromatography with AAS and ICP-MS detection. *Applied Organometallic Chemistry* **12**, 129 (1998).

88. N. Ulrich, Speciation of antimony(III), antimony(V) and trimethylstiboxide by ion chromatography with inductively coupled plasma atomic emission spectrometric and mass spectrometric detection. *Analytica Chimica Acta* **359**, 245 (1998).

89. J. Lintschinger, I. Koch, S. Serves, J. Feldmann, and W. R. Cullen, Determination of antimony species with high-performance liquid chromatography using element specific detection. *Fresenius Journal of Analytical Chemistry* **359**, 484 (1997).

90. P. Smichowski, J. Madrid, M. B. De La Calle Guntinas, and C. Camara, Separation and determination of antimony(III) and antimony(V) species by high-performance liquid chromatography with hydride generation atomic absorption spectrometric and inductively coupled plasma mass spectrometic detection. *Journal of Analytical Atomic Spectrometry* **10**, 815 (1995).

91. A. Guy, P. Jones, and S. J. Hill, Identification and chromatographic separation of antimony species with alpha-hydroxy acids. *Analyst* **123**, 1513 (1998).

92. N. Ulrich, Study of ion chromatographic behavior of inorganic and organic antimony species by using inductively coupled plasma mass spectrometric (ICP-MS) detction. *Fresenius Journal of Analytical Chemistry* **360**, 797 (1998).

93. E. M. Krupp, R. Gruemping, U. R. R. Furchtbar, and A. V. Hirner, Speciation of metals and metalloids in sediments with LTGC/ICP-MS. *Fresenius Journal of Analytical Chemistry* **354**, 546 (1996).

94. J. Feldmann, T. Riechmann, and A. V. Hirner, Determination of organometallics in intra-oral air by LT-GC/ICP-MS. *Fresenius Journal of Analytical Chemistry* **354**, 620 (1996).

95. J. Feldmann, R. Grümping, and A. V. Hirner, Determination of volatile metal and metalloid compounds in gases from domestic waste deposits with GC/ICP-MS. *Fresenius Journal of Analytical Chemistry* **350**, 228 (1994).

96. U. M. Grüter, J. Kresimon, and A. V. Hirner, A new HG/LT-GC/ICP-MS multi-element speciation technique for real samples in different matrices. *Fresenius Journal of Analytical Chemistry* **368**, 67 (2000).

2 TECHNIQUE DEVELOPMENT

2.1 DEVELOPMENT OF A SOLID-PHASE EXTRACTION METHOD FOR THE REMOVAL OF INORGANIC ANTIMONY COMPOUNDS FROM BIOLOGICAL SAMPLES

2.1.1 Introduction

It has been reported that the antimony biomethylation efficiency of *Scopulariopsis brevicaulis* is very low; with only a tiny fraction of the inorganic antimony substrate supplied being transformed to volatile and involatile methylated species.[1, 2] The quantity of volatile methylantimony species detected in culture headspace gases of mixed inoculum cultures has likewise been very low.[3-5] These reports demonstrate that not only is antimony biomethylating capability not confined to a single microbial species or genus, but also indicate that biomethylation of inorganic antimony substrate by other microbial species will likely result in the formation of only *trace levels* of methylantimony.

The high antimony substrate levels used to amend incubations are problematic during hydride-generation-GC-AAS analysis. Sodium borohydride derivatisation of culture supernatants supplied with high levels of potassium antimony tartrate or potassium hexahydroxyantimonate results in the formation of a large stibine peak. The quantities of stibine formed are significant enough that masking of the presence of any methylantimony species that may have been present in the culture supernatant could occur. It was therefore necessary to develop a simple technique that could be applied on a routine basis to reduce the levels of inorganic antimony substrate present in culture supernatants prior to hydride generation-GC-AAS analysis.

The separation methods of Smichowski *et al.* [6] and Andrewes *et al.*,[2] were adapted for the treatment of culture supernatants. Smichowski *et al.* reported 100% retention of antimony III and antimony V (100 ng) on a basic alumina column using 0.2 $mol.l^{-1}$ phosphoric acid as a mobile phase. No investigation of the conditions for the elution of methylantimony species was reported. Andrewes *et al.* also used basic alumina columns, with a mobile phase of 50 $mmol.l^{-1}$ ammonium carbonate (pH 12), to separate inorganic and methylantimony species. They reported removal of inorganic antimony

from 20 ml *S.brevicaulis* culture medium amended with an "appropriate amount of antimony compound".

Typically, culture incubations used in this study were amended with 50 $mg.l^{-1}$ inorganic antimony substrate. Coupled with the maximum analytical volume of 50 ml for the hydride generation system used for GC-AAS analysis, this meant that any separation system must be able to cope with typical inorganic antimony loadings of 2.5 mg. Detection of involatile methylantimony species in *S.brevicaulis* incubations (mineral salts medium) has been reported to be of the order of 5 $ng.ml^{-1}$ [2] (amounting to a column loading requirement of around 250 ng). Jenkins *et al.*'s (1998) detection of milligram quantities of volatile antimony species (identity not known) in culture headspace gases of *S.brevicaulis* incubations (complex organic medium) [1], suggests that these data may be an underestimation of the potential levels of methylantimony species formed in optimised culture medium.

2.1.2 Experimental

A slurry of basic alumina (Brockmann type I, 80-200 mesh) Fisher Scientific (Loughborough, Leic. UK) and mobile phase was poured into 20 ml glass syringes, plugged with glass wool and sealed at the outlet with a gate clamp, such that a final bed volume of 10 ml was achieved. The column bed was protected from disturbance by a further layer of glass wool. Test solutions (70 ml) containing 5 mg potassium hexahydroxyantimonate + 125 μg trimethylantimony oxide were passed through the alumina columns at a flow rate of 1 $ml.min^{-1}$. These loading levels were chosen (i) to be certain of achieving detection of the methylated antimony species and (ii) to test the capacity of the columns to remove a higher than normal inorganic antimony substrate loading. The first 15 ml of column eluent was discarded, subsequent eluent was collected in a clean 60 ml plastic centrifuge tube. After application of the total test volume to the column, columns were allowed to run to dryness.

Mobile phases tested were:

0.2 $mol.l^{-1}$ phosphate buffer pH 7.0

0.2 $mol.l^{-1}$ phosphoric acid (adjusted with NaOH to pH 7.5) [6]

0.1 $mol.l^{-1}$ potassium acetate pH 7.5

0.2 $mol.l^{-1}$ phosphoric acid (adjusted with NaOH to pH 9.4) [6]

0.2 mol.l^{-1} phosphate buffer pH 9.6

0.2 mol.l^{-1} carbonate buffer pH 9.6

0.1 mol.l^{-1} sodium carbonate pH 11.2 (based on)[2]

Analysis of column eluents was performed by HPLC-hydride generation-atomic fluorescence spectroscopy as detailed elsewhere in this thesis (section 4.2.17). Following HPLC analysis, the best three mobile phases in terms of potassium hexahydroxyantimonate retention and trimethylantimony oxide elution (0.2 mol.l^{-1} phosphate buffer pH 7.0, 0.1 mol.l^{-1} potassium acetate pH 7.5, and 0.1 mol.l^{-1} sodium carbonate pH 11.2) were further tested with respect to (i) retention of inorganic antimony III supplied as potassium antimony tartrate and (ii) the effect of a mixed inorganic antimony solution upon elution of trimethylantimony oxide.

Test solutions (70 ml) containing (i) 5 mg potassium antimony tartrate + 125 µg trimethylantimony oxide, (ii) 5 mg potassium hexahydroxyantimonate + 125 µg trimethylantimony oxide or (iii) 5 mg potassium antimony tartrate + 5 mg potassium hexahydroxyantimonate + 125 µg trimethylantimony oxide were applied to basic alumina columns prepared as described above. Analysis of column eluents was performed by hydride generation-GC-AAS since inorganic antimony III does not elute from the anionic exchange column under the HPLC conditions used, and also to obtain corroboration by an alternative analytical method of the HPLC data already obtained regarding the retention efficiencies of potassium hexahydroxyantimonate. GC-AAS analysis was performed as described in section 3.2.6.

2.1.3 Results

HPLC-hydride generation-AFS analysis of column eluents from test solutions containing 5 mg antimony hexahydroxyantimonate and 125 µg methylantimony oxide revealed that of the seven mobile phases tested, 0.2 mol.l^{-1} phosphate buffer pH 7.0, 0.1 mol.l^{-1} potassium acetate pH 7.5 and 0.1 mol.l^{-1} sodium carbonate pH 11.2 were the best in terms of retention of potassium hexahydroxyantimonate and elution of trimethylantimony oxide (Table 2.1). Of these mobile phases, potassium acetate and sodium carbonate were both better than the phosphate buffer mobile phase at eluting trimethylantimony oxide from the basic alumina column. Use of potassium acetate as mobile phase resulted in almost complete retention of the inorganic antimony V, and

retention of 8.9% trimethylantimony oxide; the lowest amount retained for any of the mobile phases tested. The column displayed memory effect when either 0.2 $mol.l^{-1}$ phosphoric acid pH (adjusted) 9.4 and 0.2 $mol.l^{-1}$ phosphate buffer pH 9.6 were used as column eluent (Table 2.1).

Use of phosphoric acid pH 7.5, as detailed by Smichowski *et al.*,[7] did not result in the complete retention of antimony V as reported by this group. Up to 30% of the potassium hexahydroxyantimonate eluted from the basic alumina column using this mobile phase. If a linear relationship between bed volume and retention capacity is assumed, a comparable column volume to inorganic antimony loading as detailed by Smichowski *et al.* would require a bed volume of 90 litres to ensure complete removal of 5 mg potassium hexahydroxyantimonate.

Table 2.1 HPLC-hydride generation-AFS analysis of potassium hexahydroxyantimonate and trimethylantimony oxide remaining in test solutions after passage through a basic alumina column.

Mobile phase used for elution of antimony compounds from alumina column	% trimethylantimony oxide retained on alumina column	% potassium hexahydroxyantimonate retained on alumina column
0.2 $mol.l^{-1}$ phosphate buffer pH 7.0	43.5 (10.5)	97.4 (0.6)
0.2 $mol.l^{-1}$ phosphoric acid pH (adjusted) 7.5	35.2 (11.5)	65.8 (7.6)
0.1 $mol.l^{-1}$ potassium acetate pH 7.5	8.9 (12.1)	99.3 (1.63)
0.2 $mol.l^{-1}$ phosphoric acid pH (adjusted) 9.4	54.9 (5.4)	-4.9 (9.0)
0.2 $mol.l^{-1}$ phosphate buffer pH 9.6	50.2 (14.7)	-5.0 (10.2)
0.2 $mol.l^{-1}$ carbonate buffer pH 9.6	40.8 (12.4)	43.3 (7.5)
0.1 $mol.l^{-1}$ sodium carbonate pH 11.2	19.3 (9.1)	71.2 (4.0)

Figure in parentheses are standard deviations based on three replicate samples; % retention was determined by comparison to an identical test solution that was not passed through an alumina column.

GC-AAS analysis of column eluents from test solutions containing 5 mg antimony hexahydroxyantimonate and 125 μg methylantimony oxide revealed similar data to that obtained by HPLC analysis (Table 2.2). In general, a higher degree of precision was noted using GC-AAS analysis, possibly due to the absence of hydride generation matrix effects arising from the HPLC mobile phase. Again, of the three mobile phases tested, potassium acetate was observed to be the best in terms of retention of potassium hexahydroxyantimonate on the basic alumina and elution of trimethylantimony oxide.

Table 2.2 Hydride generation-GC-AAS analysis of potassium hexahydroxyantimonate and trimethylantimony oxide remaining in test solutions after passage through a basic alumina column.

Mobile phase used for elution of antimony compounds from alumina column	% trimethylantimony oxide retained on alumina column	% potassium hexahydroxyantimonate retained on alumina column
0.2 mol.l^{-1} phosphate buffer pH 7.0	48.4 (1.8)	96.0 (1.6)
0.1 mol.l^{-1} potassium acetate pH 7.5	7.2 (1.5)	98.9 (0.1)
0.1 mol.l^{-1} sodium carbonate pH 11.2	18.4 (6.8)	72.4 (1.4)

Figure in parentheses are standard deviations based on three replicate samples; % retention was determined by comparison to an identical test solution that was not passed through an alumina column.

GC-AAS analysis of test solutions containing 5 mg potassium antimony tartrate and 125 μg trimethylantimony oxide that were passed through basic alumina columns revealed little difference in choice of mobile phase regarding retention of the inorganic antimony III compound. Use of 0.2 mol.l^{-1} phosphate buffer pH 7.0, 0.1 mol.l^{-1} potassium acetate pH 7.5 and 0.1 mol.l^{-1} sodium carbonate pH 11.2 all resulted in the almost complete retention on the column of potassium antimony tartrate (Table 2.3).

The percentages of trimethylantimony oxide retained on the basic alumina column showed a high degree of similarity when the inorganic antimony component of the test solution was supplied in either the III or the V valency state. This indicates that the presence and valency state of the inorganic antimony had little effect upon elution or retention of trimethylantimony oxide (Tables 2.1-2.3).

Table 2.3 Hydride generation-GC-AAS analysis of potassium antimony tartrate and trimethylantimony oxide remaining in test solutions after passage through a basic alumina column.

Mobile phase used for elution of antimony compounds from alumina column	% trimethylantimony oxide retained on alumina column	% potassium antimony tartrate retained on alumina column
0.2 mol.l^{-1} phosphate buffer pH 7.0	42.6 (8.9)	98.6 (0.7))
0.1 mol.l^{-1} potassium acetate pH 7.5	4.7 (3.9)	99.9 (0.3)
0.1 mol.l^{-1} sodium carbonate pH 11.2	11.2 (2.9)	99.9 (0.2)

Figure in parentheses are standard deviations based on three replicate samples; % retention was determined by comparison to an identical test solution that was not passed through an alumina column.

A high degree of similarity was observed between the retention of potassium hexahydroxyantimonate and total inorganic antimony retained when a mixed valency test solution was applied to the alumina column. This indicates that the mechanisms of association between potassium antimony tartrate and potassium hexahydroxyantimonate and the alumina column are different (Table 2.4). Smichowski *et al.* [6] reported similar findings and noted that the elution profiles of the inorganic antimony III and V compounds were not significantly different whether acidic or basic alumina was used to prepare the column. They concluded that both antimony III and V species associate with the alumina on an adsorption/desorption basis and not an anion/cation exchange mechanism.

Table 2.4 Hydride generation-GC-AAS analysis of potassium hexahydroxyantimonate, potassium antimony tartrate and trimethylantimony oxide remaining in test solutions after passage through a basic alumina column.

Mobile phase used for elution of antimony compounds from alumina column	% trimethylantimony oxide retained on alumina column	% potassium inorganic antimony retained on alumina column
0.2 mol.l^{-1} phosphate buffer pH 7.0	41.5 (9.8)	97.0 (0.6)
0.1 mol.l^{-1} potassium acetate pH 7.5	3.6 (1.8)	99.2 (0.7)
0.1 mol.l^{-1} sodium carbonate pH 11.2	9.4 (2.1)	71.8 (0.2)

Figure in parentheses are standard deviations based on three replicate samples; % retention was determined by comparison to an identical test solution that was not passed through an alumina column.

2.1.4 Conclusion

The masking effect of stibine (derived by hydride generation of inorganic antimony) on the presence of methylantimony species in culture supernatants was removed by removal of inorganic antimony from culture supernatants. Culture supernatants were passed though a basic alumina column and eluted using 0.1 mol.l^{-1} potassium acetate pH 7.5. Use of this mobile phase was found to result in negligible inorganic antimony remaining in culture supernatants (typically <0.1% potassium antimony tartrate and <1% potassium hexahydroxyantimonate of 5 mg initial column loading).

2.2 DEVELOPMENT OF A REVERSE-PHASE-HPLC TECHNIQUE FOR THE SEPARATION OF ORGANO AND INORGANIC ANTIMONY SPECIES

2.2.1 Introduction

Analysis of culture supernatants by sodium borohydride derivatisation with subsequent separation and detection of formed hydrides by gas chromatography atomic absorption spectrometry can only reveal the degree of alkylation of derivatised species. No inference can be made as to what form the alkylated substituents may take. For example, derivatisation of trimethylantimony oxide Me_3SbO and trimethylantimony chloride Me_3SbCl results, in both cases, in the formation of trimethylstibine; and derivatisation of dimethylstibinic acid ($Me_2SbH(OH)_2$), dimethylstibonous acid (Me2SbHO) and dimerised dimethylstibinic acid ($Me_2SbHO_2HSbMe_2$) (tetramethylstibinous-acid anhydride) will all result in the formation of dimethylstibine. In addition, derivatisation tends to produce several products from a single analyte (dismutation) as demethylation can be observed during the derivatisation step [8], e.g. hydride generation of trimethylantimony oxide can produce, in addition to trimethylstibine, stibine, mono- and dimethylstibine. Careful control of derivatisation conditions can limit the effect, but often, as is the case with antimony, can never be completely avoided. Separation of involatile antimony species prior to derivatisation and analysis is therefore advantageous.

The methods of HPLC separation of involatile antimony species that have been published [7, 9-11] regard separation of organic from inorganic species. In a system where the speciation of organoantimony compounds, and in particular methylantimony compounds is of interest, the reported methods are of little use since methylantimony species co-elute with the solvent front. It was also desirable to develop a HPLC separation method that could analyse microbial samples without the need for an alumina column pre-treatment stage, since this would reduce analysis times and costs.

2.2.2 Experimental

HPLC HYDRIDE GENERATION-ATOMIC FLUORESCENCE SPECTROSCOPY: GENERAL SYSTEM SET-UP

The column was connected via a six-port Rheodyne Type 50 injection valve to an Alltech 425 HPLC pump (Alltech Associates, Deerfield, Illinois, USA). The analytical column was protected by a guard column packed with the same stationary phase.

Derivatisation of metal hydrides was performed by acidifying the column eluent in a stream of acid reagent (10 $g.l^{-1}$ KI, 4 $g.l^{-1}$ ascorbic acid, 3 $mol.l^{-1}$ HCl, 4.5 $ml.min^{-1}$) with subsequent hydride generation in a stream of sodium borohydride (3% $NaBH_4$ stabilised in 0.4% NaOH, 2 $ml.min^{-1}$). Hydride generated column eluent was passed to a U-tube gas-liquid separator connected to an atomic fluorescence spectrometer (AFS) (PSA 10.003 Excalibur, PS Analytical Ltd. (Orpington, Kent, UK). Volatile antimony species were detected by an antimony specific boosted discharge hollow cathode lamp (λ = 217.6 nm). Figure 2.1 shows a schematic of the HPLC-hydride generation-AFS system. All tubing used throughout the hydride generation reaction stage comprised PTFE. It should be noted that the use of PEEK fittings for HPLC-element specific analysis of antimony is advisable since metal fittings can contribute to high background levels of antimony. Derivatised species were transported from the gas-liquid separator to the AFS by a flow of argon (200 $ml.min^{-1}$), argon was used as support gas operating in counter flow (1.7 $l.min^{-1}$) to facilitate drying of the gas before entry to the detection system. Sensitivity was increased by supplementing the carrier gas flow with a stream of hydrogen (50 $ml.min^{-1}$). AFS detector output was connected to a Hewlett Packard HP3396 series II integrator (integration parameters: attenuation, 2^5; peak width, 0.3; threshold, automatic correction).

REVERSE PHASE HPLC

Selection of mobile phase

Separations were performed using a reverse phase column; Spherisorb ODS 100 x 4.6 mm, particle size 5 μm C18 (Phenomenex, Macclesfield, Cheshire, UK). Comparison of different mobile phases was made using isocratic elution. Working solutions of antimony standards were prepared prior to analysis from concentrated stock solutions of potassium antimony tartrate (10 $g.l^{-1}$), potassium hexahydroxyantimonate (5 $g.l^{-1}$), and trimethylantimony oxide (1 $g.l^{-1}$). The mobile phases tested - methanol, acetonitrile, tetrahydrofuran, potassium acetate ± acetonitrile, disodium phosphate, and sodium dihydrogen phosphate ± acetonitrile - were prepared by dissolving the appropriate amount of each compound in millipore filtered water and buffering as required with phosphoric acid or acetic acid. In the case of organic solvents, volumes of water and solvent were dispensed separately and subsequently mixed to avoid volume contraction of the organic component. Mobile phases not containing organic solvents were degassed under vacuum in conjunction with sonication until no further release of dissolved gas

was observed. For mobile phases containing organic solvents, the aqueous phase was degassed independently prior to mixing. The column and HPLC system were conditioned with a new mobile phase for a minimum of 30 minutes prior to analysis. All mobile phases were tested at a flow rate of 0.5 $ml.min^{-1}$.

Optimisation of parameters: Reverse phase separation, mobile phase NaH_2PO_4 + acetonitrile

Use of 0.01 $mol.l^{-1}$ sodium dihydrogen phosphate + acetonitrile 5% as mobile phase was found to provide the best separation of potassium hexahydroxyantimonate and trimethylantimony oxide using reverse-phase chromatography. Complete resolution was not achieved however, therefore investigations as to the effect of molarity (1 $mmol.l^{-1}$ – 0.05 $mol.l^{-1}$) and pH (2.8 – 5.5) of sodium dihydrogen phosphate, and mobile phase flow rate (0.5 – 2.5 $ml.min^{-1}$) were made. Mobile phases were prepared as described above, and as before, the column and HPLC system were conditioned with a new mobile phase for a minimum of 30 minutes prior to analysis.

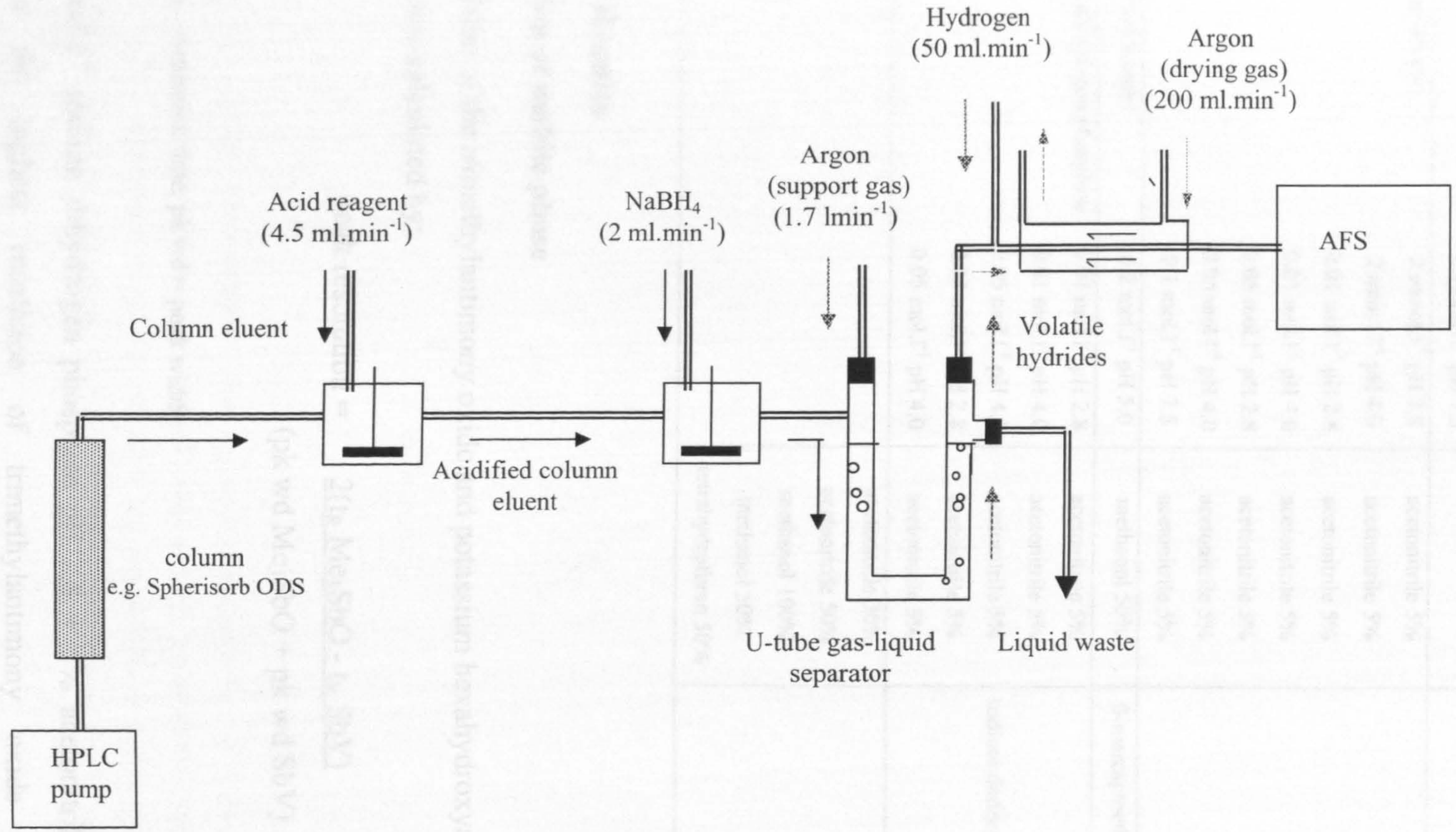


Figure 2.1 Schematic of HPLC-hydride generation-atomic fluorescence spectrometry system used for analysis of involatile antimony species in culture media of *C.humicolus* incubations.

Table 2.5 List of mobile phases tested for elution of antimony species using reverse-phase chromatography.

Aqueous solution		Organic solvent	Modifiers
Disodium hydrogen phosphate	0.01mole.l^{-1} pH 7.5		
	0.5 mol.l^{-1} pH 7.5		
	0.1mole.l^{-1} pH 7.5		
Potassium acetate	2 mmol.l^{-1} pH 2.8	acetonitrile 5%	
	2 mmol.l^{-1} pH 4.0	acetonitrile 5%	
	0.01 mol.l^{-1} pH 2.8	acetonitrile 5%	
	0.01 mol.l^{-1} pH 4.0	acetonitrile 5%	
	0.05 mol.l^{-1} pH 2.8	acetonitrile 5%	
	0.05 mol.l^{-1} pH 4.0	acetonitrile 5%	
	0.1 mol.l^{-1} pH 7.5	acetonitrile 5%	
Ammonium acetate	0.2 mol.l^{-1} pH 5.0	methanol 50%	β-mercaptoethanol (0.1 mmol.l^{-1}) [12]
Sodium dihydrogen phosphate	0.01 mol.l^{-1} pH 2.8	acetonitrile 5%	
	0.01 mol.l^{-1} pH 4.0	acetonitrile 5%	
	0.05 mol.l^{-1} pH 4.5	acetonitrile 5%	sodium dodecyl sulfate (40 mg.l^{-1}) [13]
	0.05 mol.l^{-1} pH 2.8	acetonitrile 5%	
	0.05 mol.l^{-1} pH 4.0	acetonitrile 5%	
Water		acetonitrile 30%	
		acetonitrile 50%	
		methanol 100%	
		methanol 50%	
		tetrahydrofuran 50%	

2.2.3 Results

Selection of mobile phase

Resolution of the trimethylantimony oxide and potassium hexahydroxyantimonate peaks was calculated by:

$$\text{peak resolution} = \frac{2(t_R\ Me_3SbO - t_R\ SbV)}{(\text{pk wd } Me_3SbO + \text{pk wd } SbV)}$$

where: t_R = retention time, pk wd = peak width

0.01 mol.l^{-1} sodium dihydrogen phosphate pH 2.8 + 5% acetonitrile was found to achieve the highest resolution of trimethylantimony oxide and potassium hexahydroxyantimonate. A resolution of 1.76, equating to a peak overlap of between 2 and 3% was achieved using this mobile phase at a flow rate of 0.5 ml.min^{-1} (Table 2.6). The peak resolution can be seen in Figure 2.2

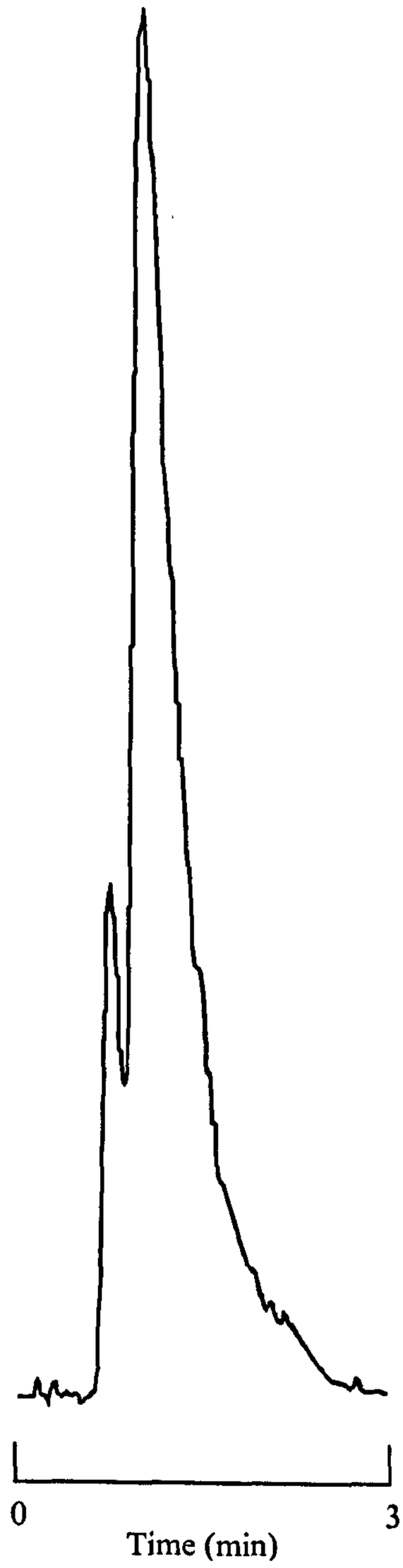


Figure 2.2 Typical chromatogram showing optimum resolution of trimethylantimony oxide and potassium hexahydroxyantimonate obtained using reverse phase phase (Spherisorb ODS) with 0.01 mol.l^{-1} sodium dihydrogen phosphate pH 2.8 + 5% acetonitrile as mobile phase. Retention times are potassium hexahydroxyantimonate 1.2 min and trimethylantimony oxide 1.7 min.

Table 2.6 Resolution of trimethylantimony oxide and potassium hexahydroxyantimonate standards using various mobile phases.

Mobile phase		Peak resolution of trimethylantimony oxide and potassium hexahydroxyantimonate
Disodium hydrogen phosphate	0.01 mol.l^{-1} pH 7.5	0.15
	0.5 mol.l^{-1} pH 7.5	0.33
	0.1 mol.l^{-1} pH 7.5	-0.08
Potassium acetate + 5% acetonitrile	2 mmol.l^{-1} pH 2.8	0.28
	2 mmol.l^{-1} pH 4.0	0.32
	0.01 mol.l^{-1} pH 2.8	0.04
	0.01 mol.l^{-1} pH 4.0	0.07
	0.05 mol.l^{-1} pH 2.8	-0.15
	0.05 mol.l^{-1} pH 4.0	-0.09
	0.1 mol.l^{-1} pH 7.5	0.06
Ammonium acetate + 50% methanol + 0.1mmole.l^{-1} β-mercaptoethanol	0.2 mol.l^{-1} pH 5.0	no elution of either species
Sodium dihydrogen phosphate + 5% acetonitrile	0.01 mol.l^{-1} pH 2.8	1.76
	0.01 mol.l^{-1} pH 4.0	0.11
	0.05 mol.l^{-1} pH 2.8	0.93
	0.05 mol.l^{-1} pH 4.5	0.04
Sodium dihydrogen phosphate + 5% acetonitrile + 40 mg.l^{-1} sodium dodecyl sulfate	0.05 mol.l^{-1} pH 4.5	0.0
Acetonitrile 30% (aq)		no elution of trimethylantimony oxide
Acetonitrile 50% (aq)		"
Methanol 100% (aq)		"
Methanol 50% (aq)		"
Tetrahydrofuran 50% (aq)		"

Optimisation of parameters: Reverse phase separation, mobile phase NaH_2PO_4 + acetonitrile

Molarity

Increasing the molarity of the mobile phase (pH 2.8) did not improve the resolution of the trimethylantimony oxide and potassium hexahydroxyantimonate peaks. The retention time of trimethylantimony oxide decreased from 1.27 min. to 0.97 min. when the mobile phase molarity was increased from 1 mmol.l^{-1} to 0.05 mol.l^{-1}. The retention time of potassium hexahydroxyantimonate remained relatively constant at 0.75 min. In terms of peak resolution, a slight increase in resolution was observed when mobile

phase molarity was increased from 1 $mmol.l^{-1}$ to 2 $mmol.l^{-1}$, and this resolution subsequently decreased as mobile phase molarity was further increased to 0.05 $mol.l^{-1}$ (Figure 2.3).

Flow rate

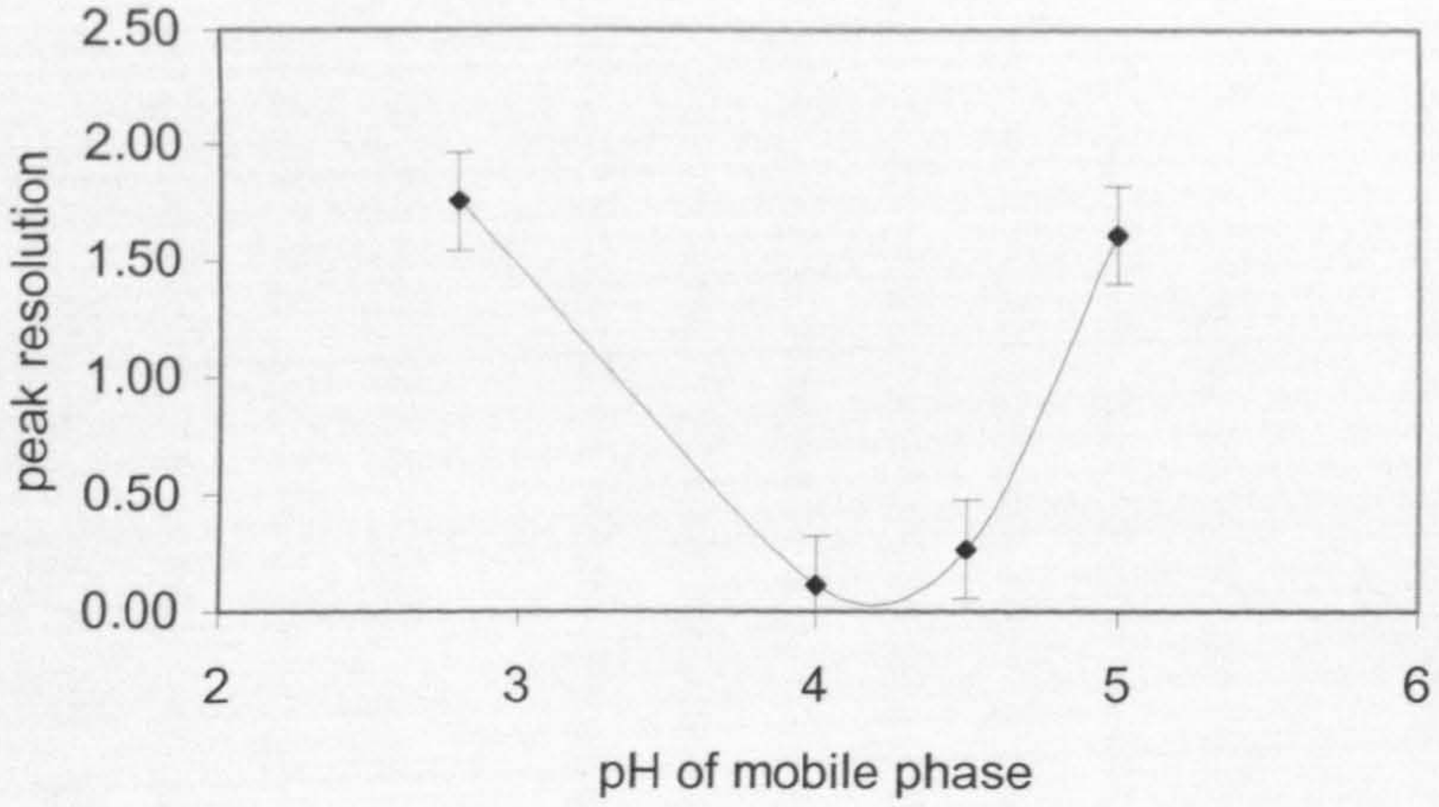
Decreasing the flow rate of the mobile phase from 2.5 to 0.5 $ml.min^{-1}$ resulted in an increase in the resolution of the two antimony species (Figure 2.3). Further decreases in flow rate were not investigated at the time. Although peak broadening was observed as flow rate was lowered, it was not significant enough at the flow rates investigated, to detrimentally impact upon peak resolution of trimethylantimony oxide and potassium hexahydroxyantimonate.

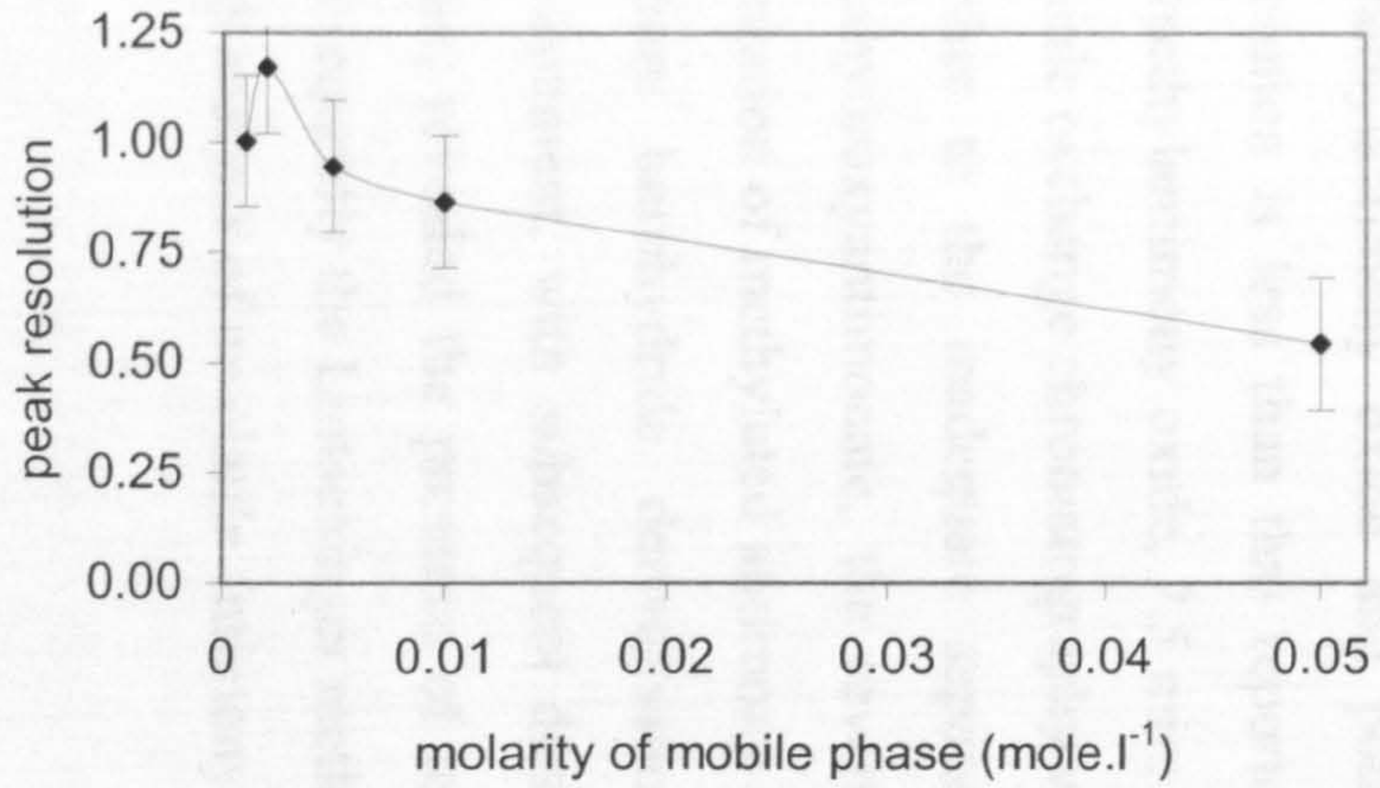
pH

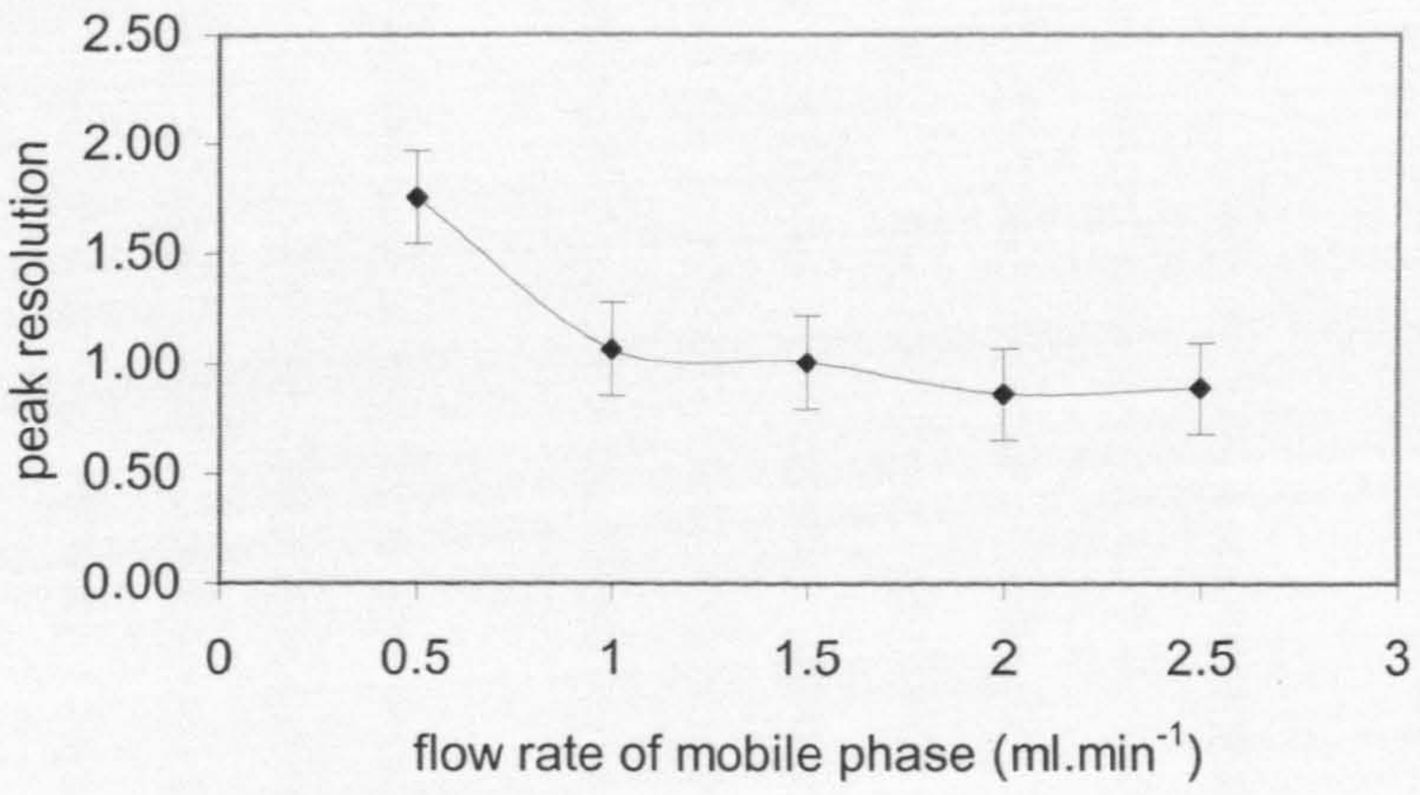
Between the pH range 2 and 10.5, oxyanions of the antimony oxides $Sb(OH)_3$, Sb_2O_4 and Sb_2O_5 do not form.[11, 14] Changing the mobile phase pH would therefore not affect the elution of these compounds. The fact that resolution of trimethylantimony oxide and potassium hexahydroxyantimonate varied over the pH range 2.8 to 5.5 indicates that the ionic nature of these compounds changes over this range. The increased resolution of trimethylantimony oxide and potassium hexahydroxyantimonate at the extremes of the pH range tested suggests that separation of these species is more dependant upon the inorganic constituent of these compounds than the organo part (Figure 2.3).

Acetonitrile

Addition of acetonitrile to the sodium dihydrogen phosphate mobile phase had a positive effect upon resolution of trimethylantimony oxide and potassium hexahydroxyantimonate when acetonitrile formed 5% of the eluent volume. Further increase in the acetonitrile content of the mobile phase however, resulted in reduced peak resolution. Percentage compositions of 8 and 10% acetonitrile resulted in coelution of the antimony species, and further elevation of the acetonitrile content to 12 and 15% resulted in increased retention of trimethylantimony oxide over the inorganic antimony V species (Figure 2.3).







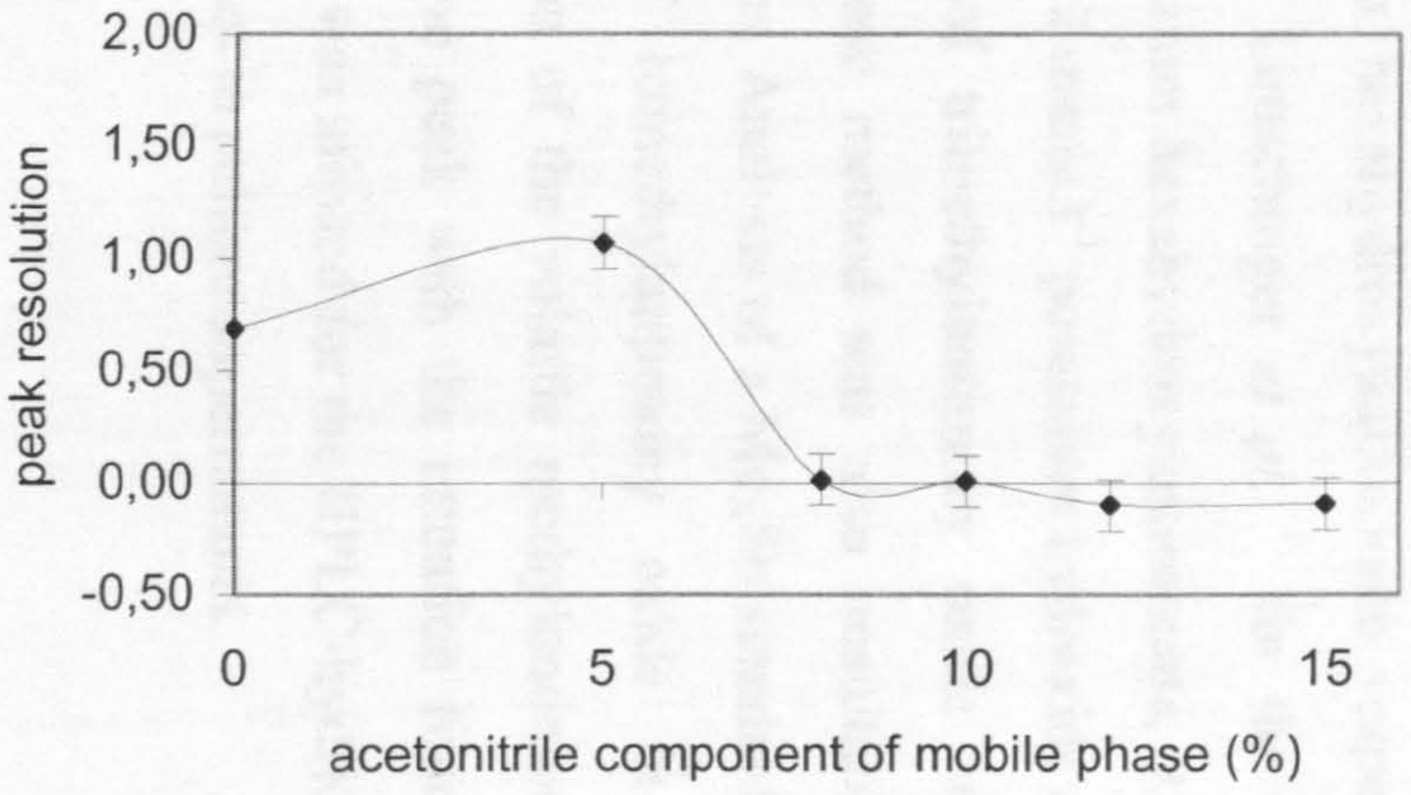


Figure 2.3 Effect of modifying pH, molarity, acetonitrile content and flow rate of sodium dihydrogen phosphate mobile phase during reverse-phase separation of trimethylantimony oxide and potassium hexahydroxyantimonate. Error bars show maximum standard deviation based on three replicate injections. Tabulated data can be found in Appendix III

2.2.4 Conclusions

A maximum resolution of 1.76 was obtained by reverse phase chromatography separation of trimethylantimony oxide and potassium hexahydroxyantimonate using a mobile phase of 0.01 $mol.l^{-1}$ sodium dihydrogen phosphate pH 2.8 + 5% acetonitrile at a flow rate of 0.5 $ml.min^{-1}$. Retention times were 2.0 min and 1.3 min for trimethylantimony oxide and potassium hexahydroxyantimonate respectively. This resolution is less than that reported by Lintschinger *et al.* [11] for the same species (trimethylantimony oxide, 7.5 min; potassium hexahydroxyantimonate, 9.2 min.) using anionic exchange chromatography with a 2 $mmol.l^{-1}$ potassium hydroxide mobile phase. Further to the inadequate separation of trimethylantimony oxide and potassium hexahydroxyantimonate, the reverse phase method was also unsuitable for HPLC separation of methylated antimony species. Analysis of a Me_XSb standard produced by sodium borohydride derivatisation of trimethylantimony oxide in an aerobic environment, with subsequent dissolution of the volatile methylantimony species in water, revealed the presence of only one peak with the retention time of 2.0 min. Consequently the Lintschinger method [11] was utilised for the HPLC-hydride generation-AFS analysis of involatile antimony species in culture supernatants.

2.3 DEVELOPMENT OF A SOLID-PHASE MICRO-EXTRACTION (SPME) TECHNIQUE FOR THE INTRODUCTION OF VOLATILE ANTIMONY SPECIES TO A GC-MS

2.3.1 Introduction

A thermal desorption unit for introduction of gaseous samples to the GC-MS was unavailable during the research period spent at the University of Canberra, Australia. Consequently, there was a need to develop an alternative method of gaseous sample pre-concentration prior to introduction to the GC injector port.

Solid-phase micro-extraction (SPME) was chosen as a suitable sample introduction method since equipment requirements were minimal and relatively low cost. Additionally, the extraction procedure was simple allowing rapid routine analysis to be performed once the system was optimised for the analysis of methylantimony species.

Solid phase microextraction gas chromatography-mass spectrometry (SPME-GC-MS) has been extensively used for the analysis of volatile organic compounds (VOC's).[15-18] SPME with GC-MS or inductively coupled plasma-mass spectrometry detection has in recent times been applied to the analysis and speciation of involatile organometallic compounds of lead, tin and mercury.[19-21] As yet, there are no reports of the analysis of volatile organometallic compounds of environmental origin using SPME.

SPME is a simple, solventless technique that allows the rapid pre-concentration of trace compounds from samples. Volatile species may be sampled from the sample headspace, whilst involatile species may be sampled from the aqueous phase, or derivatized to a volatile form and sampled from the headspace[22]. The technique excludes water from the extract, which is usually a problem when headspace and aqueous samples are extracted by other means.[23] This is advantageous for the analysis of environmental samples such as microbial cultures where abundant quantities of water are present in headspace gases.

The SPME technique relies upon the equilibration of analytes between the liquid, the headspace gas and the stationary phase coating of the fibre. Equilibration therefore depends on the dissociation constant of the analyte and the thickness of the stationary phase. The amount of analyte adsorbed by the fibre is directly proportional to the

concentration of the analyte in the sample when the system is at equilibrium. The relationship of these factors is described by the equation:

$$n = \frac{C_0 V_1 V_2 K}{K V_1 + K_2 V_3 + V_2}$$

n is the number of analyte moles sorbed by the fibre coating

C_0 is the initial analyte concentration in the aqueous phase

V_1, V_2 and V_3 are the volumes of the coating, aqueous phase and the headspace respectively

K_1 is the partition coefficient of the analyte between the headspace and the aqueous phases

K_2 is the analyte partition coefficient between the fibre coating and the aqueous phase.

SPME extraction is therefore regulated by partition coefficients and Henry's constants, which are temperature dependent [24] and the volume of aqueous sample and headspace volume.

2.3.2 Methodology

GC-MS analysis of methylantimony species

All analyses were performed on a HP 5890 gas chromatograph with a split-splitless injector, a flame ionisation detector, and a HP 5970 mass spectrometer (Hewlett Packard, Blackburn, Victoria, Australia). The injector was operated in splitless mode with the purge off for 30 seconds. Separations were performed using a 30 m x 0.25 mm, 0.25 μm film thickness, HP-35 (3% phenylmethylsilicone) capillary column (Hewlett Packard). The inlet port and detector were maintained at 250°C and 300°C respectively. Helium (3 $ml.min^{-1}$) was used as the carrier gas. Column head pressure was maintained at 70 kPa. After a sample run, the oven temperature was raised to 250°C for 20 minutes to purge any materials retained on the column. The mass spectrometer was operated in electron impact mode, monitoring positive ions. Scanning was over the range 50-250 mass units. GC injector port inlet liners of diameter of 2 mm and 4 mm id and were tested, as was the effect of initial column temperature and oven temperature during the first part of an analytical run. Cryogenic cooling of the initial part of the column was

achieved by fitting of a cold trap tee (SGE, Ringwood, Victoria, Australia) supplied with $CO_2(l)$.

Solid-phase micro-extraction

SPME was performed using polydimethylsiloxane fibres, 100 μm film thickness (Supelco, Castle Hill, New South Wales, Australia). SPME fibres were conditioned in the GC injection port for 1 hour at the start of the analytical run, and subsequently for 30 minutes between each injection. Extractions were performed at room temperature (21°C), and the sample was agitated continuously using magnetic stirring. Sensitivity of SPME sampling of analytes from headspace gases has been demonstrated to be inversely related to the headspace volume.[25, 26] The sample headspace was set at 0.5 ml for standards since this was the smallest headspace volume that could practically be used. The gauge setting on the manual fibre holder for headspace extractions was 1.8. Once the sample period was complete, the gauge setting on the manual fibre holder was adjusted to 3.0 and the fibre was transferred to the GC injector port. The extraction period was varied to optimise extraction of species of interest with respect to background noise.

Preparation of methylantimony standards

Stibine, monomethylstibine, dimethylstibine and trimethylstibine standards were prepared by hydride generating trimethylantimony dichloride (kindly donated by Prof. W.R. Cullen, University of British Columbia, Canada) in an oxygenated atmosphere to achieve demethylation of the trimethylated antimony species. A 5 ml reaction vial containing 4.5 ml acidified sample (200 μl 10% HCl, millipore water + trimethylantimony dichloride standard) was hydride generated by the addition of 250 μl 8% sodium borohydride (prepared fresh daily). The SPME fibre was exposed to the headspace of the reaction vial immediately after sodium borohydride addition. Once the extraction procedure and analysis were optimised, a calibration curve was produced by repeat injections of trimethylantimony dichloride. Sensitivity of SPME sampling of analytes from headspace gases has been demonstrated to be inversely related to the headspace volume.[25, 26] The sample headspace was set at 0.5 ml, since this was the smallest headspace volume that could practically be used.

2.3.3 Results

Effect of length of extraction period

Increasing the extraction period from 5 to 20 minutes selectively increased the amount of methylantimony analyte detected over background noise by GC-MS. Further increase in the extraction period to 40 minutes again increased the amount of methylantimony detected, but also significantly increased background noise. The background noise was identified as being siloxanes, phthalates and hexanedioc acid esters, which most likely arose from the plastic septa system used with the reaction vial. Extractions performed over a 10 hour period resulted in loss of resolution of the methylantimony peak, and extreme exacerbation of the siloxane, phthalate and hexanedioc acid ester peaks appearing later in the spectra (Figure 2.4). Extraction time is directly related to the analyte distribution constant, which generally increases with increasing molecular weight.[25] Increasing the extraction period will therefore generally result in selective enhancement of the extraction of higher molecular weight compounds, such as the plasticisers observed here.

As has been previously reported by Feldmann *et al.* [27] the spectra showed the rearrangement and migration of H atoms. The loss of H from Sb-CH_3 groups and the appearance of hydrides such as $MeSbH^+$ (m/z 137/138) and Me_2SbH^+ (m/z 152/154) were both noted. The m/z fragment 122/124 corresponding to Sb-H was also noted, despite only trimethylantimony dichloride being used as the standard.

Possible carryover between subsequent analyses, i.e. the incomplete desorption of sample analyte from SPME fibres was investigated. Analysis of consecutive fibre blanks following extraction of a 40 $mg.l^{-1}$ derivatised trimethylantimony dichloride solution did not reveal the presence of retained stibine or methylantimony species. This indicates that the volatile antimony species were efficiently desorbed from the fibre under the experimental conditions used. Cleaning of fibres between extractions was performed (20 min. at 250°C) to fully desorb recalcitrant higher molecular weight compounds, such as siloxanes and phthalates, from the fibre.

Removal of the guard column and reduction of the diameter of the GC injector port inlet liner from 4 to 2 mm i.d. both resulted in peak sharpening since the linear velocity of

the analytes through the injector port was increased and analytes were introduced onto the column in a narrower band.

Modification of temperature programming

Resolution of the derivatised products of trimethylantimony dichloride, i.e. stibine, monomethylstibine, dimethylstibine and trimethylstibine was improved by decreasing the initial temperature of the capillary column. Application of cryogenic focusing (-50°C) improved peak sharpening. Decreasing the initial column temperature programme from 5°C.min^{-1} to 0.1°C.min^{-1} after application of cryogenic focusing resulted in the resolution of all four volatile antimony peaks (Figure 2.5). Retention times were stibine 1.3 min, monomethylstibine 1.5 min, dimethylstibine 1.8 min and trimethylstibine 2.1 min.

Optimised procedure

SPME fibres were conditioned by heating the exposed fibre in the GC injector port (250°C) for one hour at the start of an analytical run and subsequently for 30 minutes between each injection. Extraction of volatile antimony species from culture headspace gases was performed at room temperature (21°C) for 20 minutes. The sample was agitated continuously using magnetic stirring. To avoid unwarranted derivatisation of antimony species by trace contamination of sodium borohydride, reaction vessels used for the preparation of volatile antimony standards from trimethylantimony dichloride were not used for the analysis of culture headspace gases. The gauge setting on the manual fibre holder was 1.8 during the extraction period. Once the extraction period was complete, the fibre was retracted and the gauge setting on the manual fibre holder was adjusted to 3.0. The fibre was immediately transferred to the GC injector port for analysis. Samples were cold trapped and cryogenically focused by cooling the initial part of the column to -50°C. Oven temperature was programmed such that oven temperature was maintained at 30°C for 2 minutes, and then ramped to 36°C at 0.1°C.min^{-1} and held at this temperature for a further 2 minutes. After each sample was analysed the temperature of the oven was raised to 250°C for 20 minutes to purge any materials retained on the column.

A linear calibration of equation $y = 4.898x-1.8339$ ($R^2 = 0.978$) was obtained for trimethylantimony dichloride loading and total amount of derivatised volatile antimony species detected by GC-MS. The ratio of volatile antimony species (stibine, monomethylstibine, dimethylstibine and trimethylstibine) was observed to be constant over the concentration range of trimethylantimony dichloride studied. At 15 ng trimethylantimony dichloride loading, derivatised stibine, monomethylstibine, dimethylstibine and trimethylstibine were all observed. Trimethylstibine could be reliably detected at a loading of 3.5 ng trimethylantimony dichloride. Below this loading, methylantimony peaks were lost in background noise.

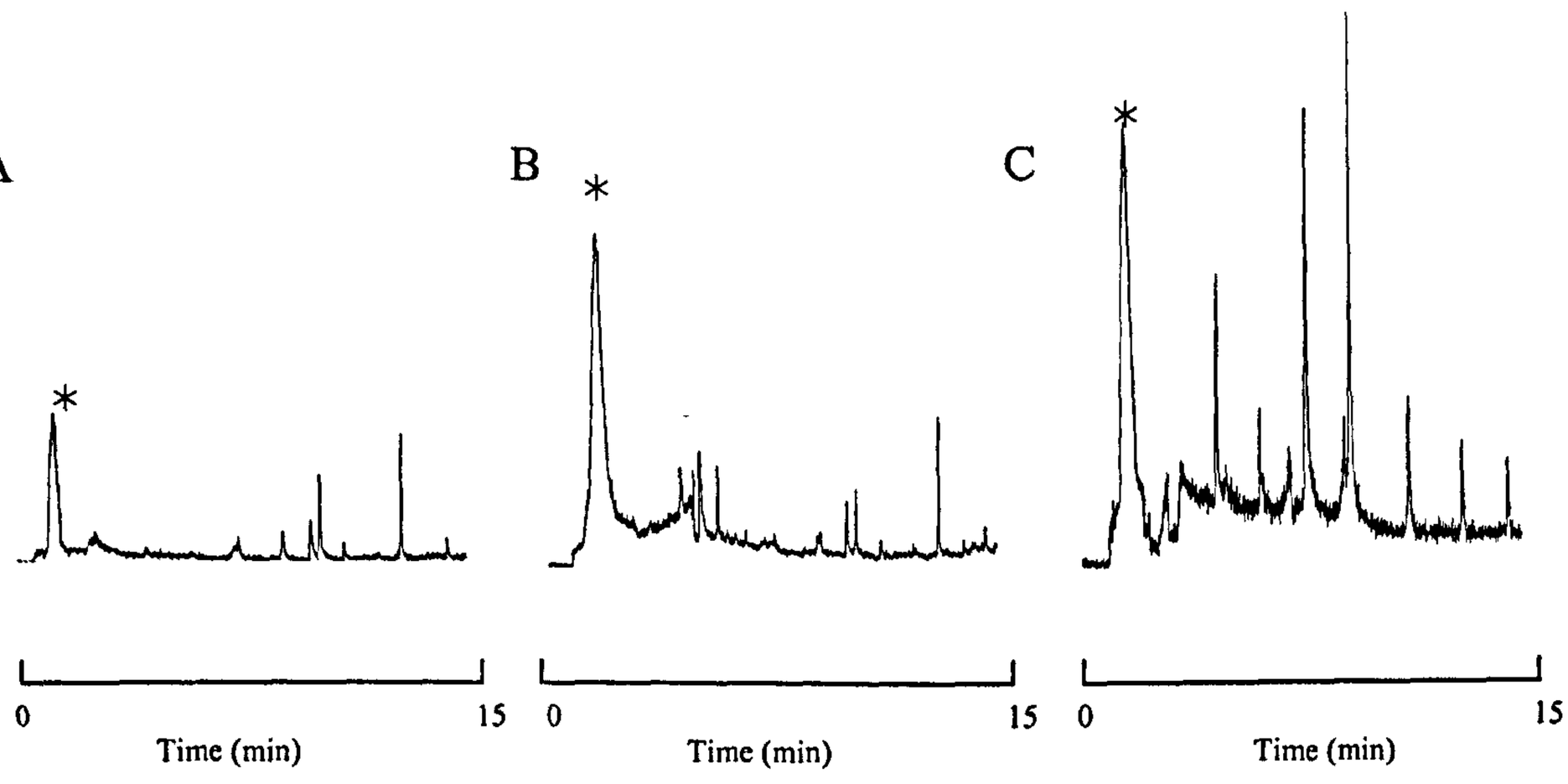


Figure 2.4 SPME-GC-MS total ion chromatograms demonstrating effect of extraction time upon extraction of trimethylstibine from headspace of a reaction vial containing sodium borohydride derivatised trimethylantimony dichloride. Extraction periods are (A) 5 minutes, (B) 20 minutes and (C) 40 minutes. The peak marked * represents trimethylstibine.

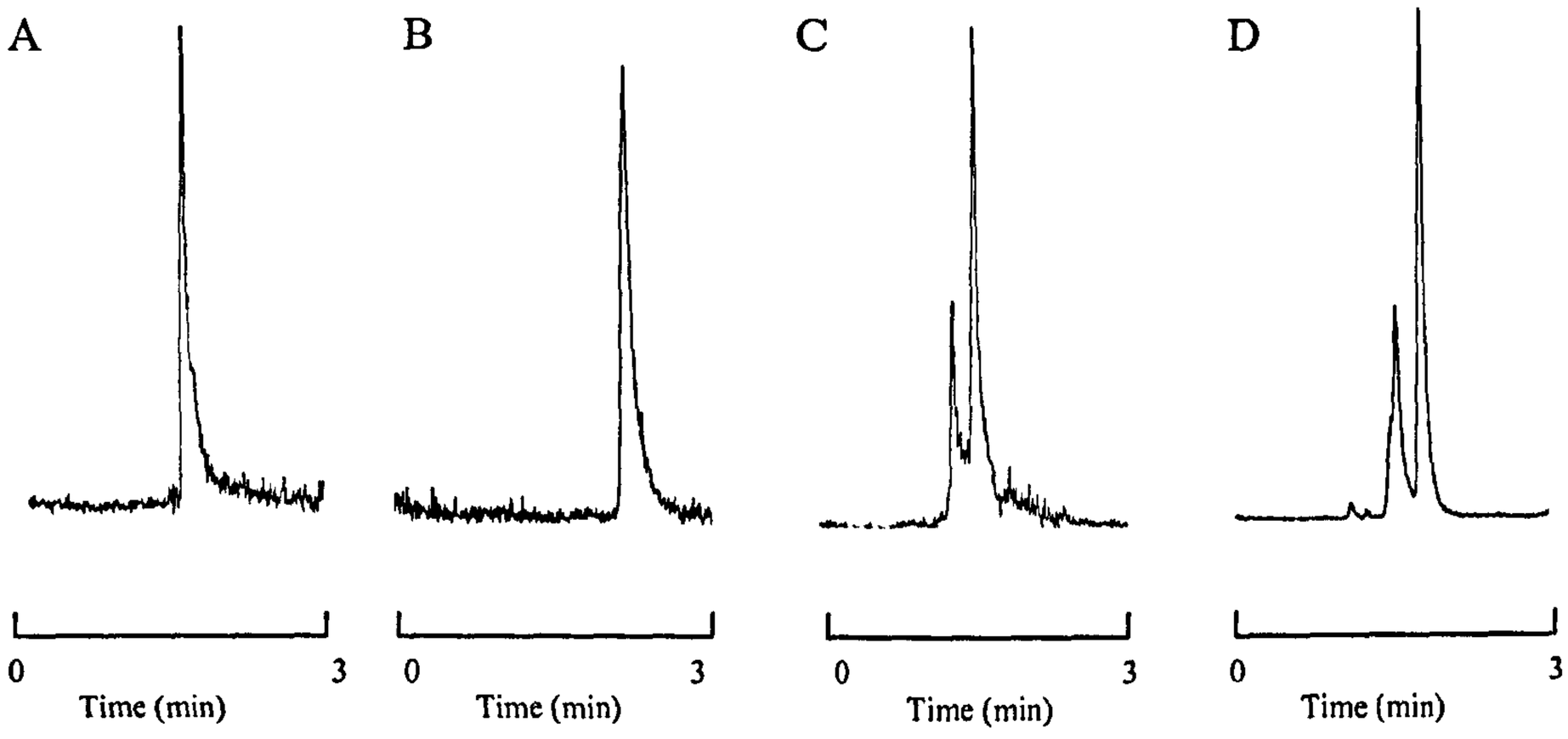


Figure 2.5 SPME-GC-MS total ion chromatograms of volatile methylantimony species produced by sodium borohydride derivatisation of trimethylantimony dichloride demonstrating the effect of initial column temperature and subsequent temperature ramp upon peak shape and resolution. The column was held at initial temperature for 2 minutes and then elevated to the final temperature at the ramp rate shown. (A) 40°C, 10°C.min^{-1}: (B) 35°C, 5°C.min^{-1}: (C) -50°C, 1.0°C.min^{-1}: (D) -50°C, 0.1°C.min^{-1}. Refer to figure 2.6 for identity of peaks.

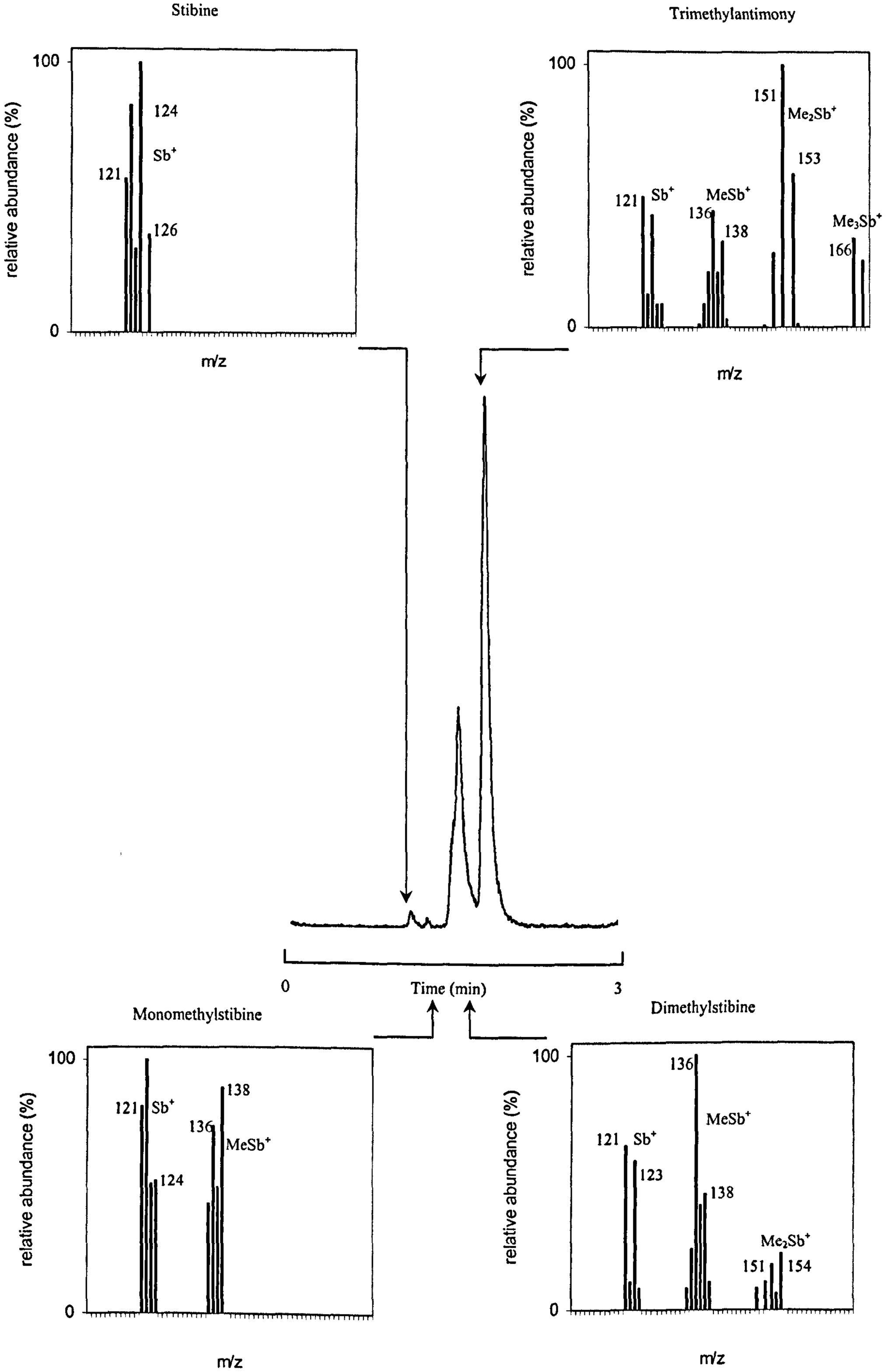


Figure 2.6 Typical GC-MS fragmentation patterns of SPME extracted volatile antimony species produced by sodium borohydride derivatisation of trimethylantimony dichloride.

References for Chapter 2

1. R. O. Jenkins, P. J. Craig, W. Goessler, D. Miller, N. Ostah, and K. J. Irgolic, Biomethylation of inorganic antimony compounds by an aerobic fungus: *Scopulariopsis brevicaulis*. *Environmental Science and Technology* **32**, 882 (1998).

2. P. Andrewes, W. R. Cullen, J. Feldmann, I. Koch, P. E., and K. J. Reimer, The production of methylated organoantimony compounds by *Scopulariopsis brevicaulis*. *Applied Organometallic Chemistry* **12**, 827 (1998).

3. P. N. Gates, H. A. Harrop, J. B. Pridham, and B. Smethurst, Can microorganisms convert methylated antimony trioxide or potassium antimonyl tartrate to methylated stibines? *Science of the Total Environment* **205**, 215 (1997).

4. H. Gürleyük, V. Van Fleet-Stalder, and T. G. Chasteen, Confirmation of the biomethylation of antimony compounds. *Applied Organometallic Chemistry* **11**, 471 (1997).

5. R. O. Jenkins, P. J. Craig, D. P. Miller, L. C. A. M. Stoop, N. Ostah, and T.-A. Morris, Antimony biomethylation by mixed cultures of micro-organisms under anaerobic conditions. *Applied Organometallic Chemistry* **12**, 449 (1998).

6. P. Smichowski, M. Beatriz de la Calle, Y. Madrid, M. G. Cobo, and C. Camara, Speciation and preconcentration of Sb(III) and Sb(V) on alumina using phosphoric acid under pH-controlled conditions. *Spectrochimica Acta* **49B**, 1049 (1994).

7. P. Smichowski, J. Madrid, M. B. De La Calle Guntinas, and C. Camara, Separation and determination of antimony(III) and antimony(V) species by high-performance liquid chromatography with hydride generation atomic absorption spectrometric and inductively coupled plasma mass spectrometic detection. *Journal of Analytical Atomic Spectrometry* **10**, 815 (1995).

8. M. Dodd, S. L. Grundy, K. J. Reimer, and W. R. Cullen, "Methylated antimony compounds-synthesis, hydride generation properties and implications for aquatic speciation," p. 207. 1992.

9. J. Lintschinger, O. Schramel, and A. Kettrup, The analysis of antimony species by using ESI-MS and HPLC-ICP-MS. *Fresenius Journal of Analytical Chemistry* **361**, 96 (1998).

10. N. Ulrich, Speciation of antimony(III), antimony(V) and trimethylstiboxide by ion chromatography with inductively coupled plasma atomic emission spectrometric and mass spectrometric detection. *Analytica Chimica Acta* **359**, 245 (1998).

11. J. Lintschinger, I. Koch, S. Serves, J. Feldmann, and W. R. Cullen, Determination of antimony species with high-performance liquid chromatography using element specific detection. *Fresenius Journal of Analytical Chemistry* **359**, 484 (1997).

12. M. Hempel, H. Hintelmann, and R. D. Wilken, Determination of organic mercury species in soils by high-performance liquid chromatography with ultraviolet detection. *Analyst* **117**, 669 (1992).

13. H. M. A. Killa and D. L. Rabenstein, Determination of selenols, diselenides, and selenenyl sulfides by reversed-phase liquid chromatography with electrochemical detection. *Analytical Chemistry* **60**, 2283 (1988).

14. D. G. Brookins, "Eh-pH diagrams for geochemistry," p. 176. Springer-Verlag, Berlin, 1988.

15. M. Y. Jia, J. Koziel, and J. Pawliszyn, Fast field sampling/sample preparation and quantification of volatile organic compounds in indoor air by solid-phase microextraction and portable gas chromatography. *Field Analytical Chemistry and Technology* **4**, 73 (2000).

16. D. J. Tranthim-Fryer, R. C. Hansson, and K. W. Norman, Headspace/solid-phase microextraction/gas chromatography-mass spectrometry: A screening technique for the recovery and identification of volatile organic compounds (VOC's) in postmortem blood and viscera samples. *Journal of Forensic Sciences* **46**, 934 (2001).

17. J. Dermietzel and G. Strenge, Determination of VOC contamination in borehole sediments by headspace-SPME-GC analysis. *Fresenius Journal of Analytical Chemistry* **364**, 645 (1999).

18. M. Llompart, K. Li, and M. Fingas, Headspace solid phase microextraction (HSSPME) for the determination of volatile and semivolatile pollutants in soils. *Talanta* **48**, 451 (1999).

19. Y. Cai, S. Monsalud, K. G. Furton, R. Jaffe, and R. D. Jones, Determination of methylmercury in fish and aqueous samples using solid-phase microextraction followed by gas chromatography atomic fluorescence spectrometry. *Applied Organometallic Chemistry* **12**, 565 (1998).

20. N. G. Orellana-Velado, R. Pereiro, and A. Sanz-Medel, Solid phase microextraction gas chromatography-glow discharge- optical emission detection for tin and lead speciation. *Journal of Analytical Atomic Spectrometry* **16**, 376 (2001).

21. M. S. Fragueiro, F. Alava-Moreno, I. Lavilla, and C. Bendicho, Determination of tetraethyllead by solid phase microextraction- thermal desorption-quartz furnace atomic absorption spectrometry. *Journal of Analytical Atomic Spectrometry* **15**, 705 (2000).

22. L. Zhang and D. Planas, Biotic and abiotic mercury methylation and demethylation in sediments. *Bulletin of Environmental Contamination and Toxicology* **52**, 691 (1994).

23. B. Jaillais, V. Bertrand, and J. Auger, Cryo-trapping/SPME/GC analysis of cheese aroma. *Talanta* **48**, 747 (1999).

24. N. Cardellicchio, S. Giandomenico, A. Decataldo, and A. Di Leo, Speciation of butyltin compounds in marine sediments with headspace solid phase microextraction and gas chromatography- mass spectrometry. *Fresenius Journal of Analytical Chemistry* **369**, 510 (2001).

25. Supelco, Solid phase microextraction: Solventless sample preparation for monitoring flavour and fragrance compounds by capillary gas chromatography. Supelco, Bellefonte, Philadelphia, (1995).

26. Supelco, Monitor organic volatile impurities (OVIs) in pharmaceutical products, using solid phase microextraction/capillary GC. Sigma Aldrich, Bellefonte, Philadelphia, (1997).

27. J. Feldmann, I. Koch, and W. R. Cullen, Complementary use of capillary gas chromatography-mass spectrometry (ion trap) and gas chromatography-inductively coupled plasma mass spectrometry for the speciation of volatile antimony, tin and bismuth compounds in landfill and fermentation gases. *Analyst* **123**, 815 (1998).

3 ANTIMONY BIOMETHYLATION BY ANAEROBIC BACTERIA- MONOSEPTIC AND MIXED SOURCE INOCULA

3.1 Introduction

A number of reports of antimony biomethylation by mixed cultures of anaerobic bacteria have been detailed.[1-3] No attempt was made however, to identify single species capable of methylation from within the mixed cultures. Volatilisation of antimony from municipal waste dumps [4, 5] and from environmental sediments [6] also suggests that certain anaerobic or facultatively anaerobic bacteria may possess the capability to biomethylate antimony.

Hirner *et al.* [7] indicated that the most important criteria for a high biomethylation potential are; (i) anaerobic atmosphere (at least on a microscale), reducing conditions and slightly acidic pH values in the hydrosphere; (ii) sufficient concentrations of metal(loid)s in easily accessible forms (e.g. as ions); (iii) presence of microorganisms that are capable of metal(loid) biomethylation; and (iv) presence of transferable methyl groups. A methanogenic environment fulfils at least three of the four criteria (metal concentration obviously being variable between locations). The detection of trimethylstibine in methanogenic environments [4, 8] and the identification of trimethylstibine in headspace gases of enrichment cultures using samples from methanogenic environments as inoculum source [2, 3] may indicate that methanogenic environments are of primary importance regarding location of metal biomethylating capability.

Methanogenic environments comprise complex populations that interact synotrophically, facilitating interspecies hydrogen transfer. Methanogenic bacteria are the ultimate consumers within a methanogenic environment, producing methane from hydrogen and a restricted number of C-1 carbon compounds. Hydrolysis and fermentation of complex organic polymers by cellulolytic and other hydrolytic bacteria, and fermentative bacteria, plus acetogenesis from carbon dioxide and hydrogen by homacetogenic bacteria, is required to produce sufficient flux of these substrates.

Recently, Michalke *et al.* [9] reported the biomethylation of inorganic antimony by pure cultures of three methanogenic archaea species, of the proteolytic bacteria *Clostridium collagenovorans* and of the sulfogen *Desulfovibrio vulgaris*. Volatile trimethylstibine

was detected as the sole methylantimony species detected in culture headspace gases of these cultures. These authors did not report, however, on the possible presence of involatile mono-, di- and trimethylantimony species, such as dimethylstibinic acid, dimethylstibonous acid and trimethylantimony oxide, in the liquid culture media of pure cultures. These species could be formed as intermediates, or as final biotransformation products, of a biomethylation pathway analogous to that established for biomethylation of inorganic arsenic by certain fungi and eubacteria,[10] i.e. the Challenger mechanism.[11]

Initial work applied GC-MS to the identification of biogenic volatile antimony species and to simultaneous characterisation of microbial metabolism in samples from a variety of different microbial habitats. This approach involved purge and trap collection of headspace gases from microbial enrichment cultures, with subsequent thermal desorption and GC-MS analysis. Interpretation of the range of volatile products (sulphides, alcohols, esters, organic acids) facilitated the identification of various modes of anaerobic microbial metabolism in enrichment cultures. The relative ease of this procedure enabled rapid screening of environmental samples for the capability to biomethylate antimony. Based on the GC-MS profiles of culture headspace gases from enrichment cultures that produced trimethylstibine various *Clostridium* spp. (which are known to participate in the multistage process of methanogenesis in nature through fermentation or acetogenesis) were selected for studies in monoseptic cultures. Of the four clostridia species tested, methylated antimony compounds were detected in culture incubations from three species. In addition, two clostridia species were isolated from enrichment cultures of environmental samples that were positive for the detection of trimethylstibine. Both were demonstrated to possess an antimony biomethylating capability.

3.2 Experimental

3.2.1 Collection of soil samples

Six soil samples from diverse locations were chosen as being representative of a number of microbial habitats and therefore nutritional type. All samples were collected from a depth of 5-10 cm below the surface using a stainless-steel corer, with the exception of sample 3, which was directly sampled with the corer. Samples were stored in sterile screw capped glass bottles. The glass bottles were filled to the top to exclude O_2, and were stored in the dark at room temperature till enrichment cultures were prepared. The samples were taken from various locations in Leicestershire and Lincolnshire, UK. Sample 1 was collected from the watershed (run-off) of a busy road. Sample 2 was collected from a former urban industrial site (not connected with the use of heavy metals). Sample 3 comprised pond sediment taken from a public city park. Sample 4 was collected from a rural field, sample 5 comprised soil sludge associated with vegetation, and was taken from the watershed of a country road, and sample 6 was collected from a garden compost heap.

Total levels of antimony, arsenic, cadmium, chromium, lead, mercury and tin in the soil samples were determined using inductively coupled plasma mass spectrometry (ICP-MS) (Walter Gössler, Karl Franzens Universität, Graz). 6.5 ml of HNO_3 (conc.) was added to 3 g samples of soil and heated in a microwave for 3 cycles of 30 s at full power (650 W). After cooling samples were diluted up to a final volume of 30 ml with demineralised water and filtered to remove debris (0.22 μm pore size), giving a final concentration of 15% HNO_3 for analysis. Solutions (1 in 10 dilution) were delivered (0.95 $ml.min^{-1}$) to a Meinhard TR-30-A3 nebuliser. The resulting aerosol was subsequently routed through a double-pass Scott-type spray chamber held at 2°C before introduction to a Plasma Quad 2 Turbo Plus ICP-MS (VG Elemental, Cheshire, UK.) No attempt was made to speciate metal forms present in the soil.

3.2.2 Preparation of enrichment cultures

Soil inocula was prepared using the method of Honschopp *et al.*,[12] i.e. 10 g soil in 75 ml sterile demineralised water and incubation at 30°C and 250 rpm for 30 minutes. Aliquots (1 ml) of soil inocula were transferred to each of three different culture media; (1) denitrifying medium (DM medium),[1] (2) cooked meat medium (Oxoid, Unipath Ltd., Basingstoke, UK), (3) fecult A medium.[13] All media were prepared using a modified

Hungate technique as described by Miller and Wolin.[14] Medium (90 ml) was dispensed to 100 ml reaction vials. All vials were crimp sealed with PTFE lined, butyl rubber septa. Potassium antimony tartrate was used to amend cultures to a level of 50 $mg.l^{-1}$ antimony. All additions were made via needle and syringe through the septa. Control cultures were set up using non-inoculated media and media inoculated with sterile (autoclaved twice for 15 minutes at 121°C, with an intermediate outgrowth period of 24 hours) soil inocula. Cultures were incubated in the dark at 28°C for between 4 and 6 weeks.

3.2.3 Isolation of clostridia from enrichment cultures

Loops of culture broth from cooked meat cultures of urban wasteland soil that were positive for the detection of trimethylstibine, were streaked out on reinforced clostridial agar (RCA) + 5% agar (Oxoid, Unipath Ltd., Basingstoke, UK) and incubated in an anaerobic jar at 28°C for 2 days. An anaerobic atmosphere was generated within the anaerobic jar using a CO_2 enriched, gas generating kit (Oxoid). Discrete colonies were transferred to fresh RCA plates and re-incubated. This procedure was repeated to ascertain that the cultures were monoseptic. Two isolates were obtained using this procedure. Gram staining and microscopic examination revealed the isolates to be Gram-positive rods. The isolates were tested for catalase activity, a distinguishing feature between clostridia and the genus *Bacillus*. This test was negative, indicating that the isolates were clostridia. Identification to species level was not pursued.

3.2.4 Monoseptic cultures of anaerobic bacteria

Monoseptic cultures of all bacteria used, except for *Clostridium sporogenes* and isolates SI-1 and SI-2, were obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, UK); *Clostridium acetobutylicum* NCIMB 619, *Clostridium butyricum* NCIMB 7423, *Clostridium cochlearium* (formerly *Clostridium lentoputrescens*) NCIMB 10629. *Clostridium sporogenes* was obtained from the culture collection at De Montfort University, Leicester, UK; soil isolates SI-1 and SI-2 were isolated from an enrichment culture shown to be positive for the production of trimethylstibine. The cultures were maintained in cooked meat medium under liquid paraffin. Cooked meat medium was also used for cultivation, and was prepared as described for enrichment cultures. Fresh clostridia culture (0.5 ml) was used as inoculum. Cultures were sacrificed after 2 days, 1 week, 2 weeks, and 4 weeks. A total of 8 flasks were assayed at each time point.

3.2.5 GC-MS analysis of headspace gases

Culture headspace gases were transferred under a flow of oxy-free nitrogen (20 ml.min^{-1}, 30 min) through traps (6 cm x 5 mm) packed with 0.3 g Tenax®-TA (60/80 mesh) and subsequently analysed by (GC-MS). GC-MS analysis was performed on a Carlo Erba 8000 gas chromatograph with a split-splitless injector and a MD800 mass spectrometer (Fisons, Loughborough, UK). Volatile compounds were released from the Tenax®-TA traps by thermal desorption. Traps were heated in a TD4 (Perkin Elmer, Beaconsfield, Bucks., UK) at 200°C for 10 minutes and cryofocussed at –40°C. Volatiles were subsequently transferred under a flow of helium (3 ml.min^{-1}) via a transfer line held at 180°C. Separations were performed using a 30 m x 0.32 mm, film thickness 0.25 μm OV1 column. The inlet port and detector were maintained at 150°C and 280°C respectively, helium was used as carrier gas throughout (2 ml.min^{-1}). The column was held at 40°C for 2 minutes and then ramped at a rate of 15°C.min^{-1} to a final temperature of 150°C and held at this temperature for a further 2 minutes. The oven was heated to 250°C for 10 minutes after each analysis to remove unwanted material from the column. The mass spectrometer was operated in electron impact mode, monitoring positive ions. Scanning was over the range 40-200 mass units. Identification was based on the National Institute of Standards and Technology (NIST) library database of mass spectra. The retention time of trimethylstibine was determined by hydride generation of trimethylantimony dichloride standard to Tenax®-TA, and analysis as described for samples; retention time = 2.35 min.

3.2.6 Hydride generation-GC-AAS analysis of involatile antimony species in culture supernatants and soil inocula

Samples of soil inocula and media controls (no biomass, no antimony substrate) were analysed for the presence of pre-formed methylantimony species by hydride generation gas chromatography atomic absorption spectrometry (GC-AAS)[15]. Detection of volatile antimony species was by a Perkin Elmer (Beaconsfield, Bucks., UK) PE1300 atomic absorption spectrometer, fitted with a quartz furnace (80 mm x 120 mm) heated to 850°C–950°C and an antimony specific hollow cathode lamp (Cathodeon Ltd. Cambs., UK) λ = 217.6 nm, I = 15–25 mA, spectral bandpass = 0.7 nm, integration = 0.1 s. Sensitivity was increased by passing hydrogen (240 ml.min^{-1}) and air (30 ml.min^{-1}) into the furnace. (Nitrogen (1.6 ml.min^{-1}) was used as a make-up gas.) Samples, diluted to a final volume of 50 ml, were acidified to pH 2.4 with HCl and batch hydride generated by the addition of 250 μl 8% sodium borohydride. Prior to hydride generation samples

were deoxygenated with helium (60 ml.min^{-1}) for 10 minutes. Volatile species produced by hydride generation were cryofocussed on a chromatographic column (50 cm x 4 mm i.d. glass wound with Ni-Cr resistance wire (80/20%) (10.04 Ω.m^{-1})) packed with 10% OV101 on Chromosorb-W-HP (80/100 mesh) (Alltech Associates, Deerfield, Illinois, USA). Cooling of the trap was achieved by submersion in liquid nitrogen. Hydride generation and continuous purging of volatiles was carried out for 3 minutes, after which time the helium flow was switched so as to bypass the reaction vessel. Following the removal of the liquid nitrogen from the trap the column was heated electrothermally by constant voltage application of 12 V, to a final temperature of 90°C, during which time volatile compounds were eluted according to their boiling points. Chromatograms were produced using Peak Simple II software (SRI Instruments, Torrance, California, USA). Identification of volatile antimony species was based on comparison with the retention time of antimony standards, produced by hydride generation of trimethylantimony dichloride (typically 50-200 µl of a 10 mg.l^{-1} solution) (retention times; stibine 0.79 min, monomethylstibine 1.33 min, monomethylstibine 1.71 min, trimethylstibine 1.94 min). The order of elution and the absolute identification of these compounds were confirmed by GC-MS using the same column.

The high inorganic antimony substrate levels used to amend cultures were found to be problematic during hydride generation GC-AAS analysis as the hydride generated product of the inorganic antimony used as biotransformation substrate (SbH_3) masked the presence of methylantimony peaks. Inorganic antimony was removed from samples as described in section 2.1. i.e. culture broth was centrifuged, 4000 rpm, 15 mins, to remove biomass. Supernatant was subsequently passed through columns of basic alumina (10 ml bed volume) using 0.1 mol.l^{-1} potassium acetate, pH 7.5 as eluent. Flow rate of supernatant through the column was 1 ml.min^{-1}. The problem of samples foaming over in the reaction vessel as a result of protein degradation during hydride generation was overcome by the addition of 500 µl 10% polypropylene glycol (MW= 1080). This had the benefit of allowing large sample volumes to be hydride generated and hence avoided the need for a pre-concentration step.

3.2.7 Cyclic voltammetry

Staircase cyclic voltammetry experiments were carried out using the Autolab PGSTAT30 (Windsor Scientific Ltd., Slough, UK) with scan rate of 30 $mV.s^{-1}$ between –0.6 V and 0.6 V against a saturated calomel electrode (SCE) (0.241 V *vs.* H_2). The electrochemical cell used consisted of one compartment connected with a Luggin capillary for the reference. Platinum foil was used as a working electrode and platinum mesh as counter electrode. Addition of supporting electrolyte was not necessary as the salt content of the culture medium ensured a reversible system; redox potential was measured as the mid-point. The samples of culture (20 ml) were maintained under anoxic conditions by a flow of oxy-free nitrogen during the redox process. The voltammograms obtained were analysed using the General Purpose Electrochemical System (GPES) software, version 4.6. (Eco Chemie, Windsor Scientific, Slough, UK).

3.3 Results

3.3.1 GC-MS analysis of headspace of undefined mixed cultures

GC-MS was used to characterise the major modes of metabolism within enrichment cultures. The range of volatile compounds identified in culture headspace gases is detailed in Table 3.1. No quantification of methylantimony was carried out as GC-MS analysis was used merely as a probe of biomethylating capability.

Table 3.1 GC-MS analysis of culture headspace gases in undefined, mixed inocula enrichment cultures.

PRODUCTS	MEDIUM		
	COOKED MEAT	FECULT A	DENITRIFYING
MIXED ACID FERMENTATION			
Acetate	+	-	-
Ethanol	++	++	+
PROPIONATE FERMENTATION			
Propionate	+	+	+
SOLVENT FERMENTATION			
Acetate	++	-	-
Acetone	+	-	+
Ethanol	++	++	+
Isopropanol	+	++	-
Butanol	++	++	+
Butyrate	++	+	-
Pentanol	+	-	-
PROTEOLYTIC DIGESTION PRODUCTS			
Methylsulphides	+++	+	-
Methyl alkanethiols	+	-	-
Thiane & Thiolanes	+	-	-
S-methylethanethioate	++	+	+
S-methylbutanethioate	++	-	-
di-methylbutanethioate	+	-	-
VOLATILE ANTIMONY COMPOUNDS			
Trimethylstibine	+	-	-

Media were supplemented with 50 $mg.l^{-1}$ antimony as potassium antimony tartrate; relative quantification based on TIC for each compound; - = not detected. + = detection of compound (+++ > ++ > +)

For the cooked meat enrichment condition, the fermentation end products and wide range of sulfur compounds detected is consistent with proteolytic digestion of meat by

clostridia. In addition, the detection of butanol and acetone is indicative of the presence of butyric acid clostridia.

Fermentation end products were also detected in the headspace of Fecult A enrichment cultures, indicating the presence of both formic acid (i.e. mixed acid and butanediol) and butyric acid fermentative organisms. However, butanediol was not detected, suggesting that of the two groups of formic acid fermentative bacteria, mixed acid predominate. The range and quantity of sulfur containing compounds was not as extensive as that identified in cooked meat medium, suggesting that proteolytic clostridia form a less significant component of the population in this medium. The absence of acetate in the headspace of Fecult A enrichment cultures could be indicative of the absence of mixed acid fermentative bacteria, however this is considered to be unlikely as this medium is specifically designed to promote the growth of formic acid and butyric acid bacteria. The apparent absence of acetate may reflect its conversion to carbon dioxide and methane by methanogenic bacteria; a highly specialised group of bacteria that can only utilise hydrogen as an electron source and carbon sources such as carbon dioxide, acetate, formate, or methanol. Jenkins *et al.* [16] detected relatively large quantities of methane in conjunction with the absence of acetate in headspaces of mixed bacterial cultures using this medium, concluding that this was due to the activity of methanogenic bacteria. The detection of acetate in cooked meat cultures does not conversely indicate the absence of a methanogenic population, only that the potential population is not sufficiently large enough to utilise all the acetate available as substrate. The absence of acetone is indicative of the absence of *C.acetobutylicum*, however it is also consistent with its oxidation to isopropanol by *C.butyricum*. The detection of butanol in the culture headspace adds further weight to this supposition. Low levels of fermentation end products were identified in the headspace of cultures grown in nitrate-reduction medium, indicating that some formic acid or heterolactic fermentative growth is occurring; either by fermentation of glycerol or through secondary fermentation of lysed cells arising through growth by nitrate reduction. The primary mode of metabolism in these vials was probably anaerobic respiration using nitrate as the terminal electron acceptor. Ethanol was detected in culture headspace gases for all enrichment conditions, however being an end product of several classes of fermentation - alcoholic, heterolactic, formic acid, and butyric acid - its presence is not discriminatory.

Based on the GC-MS profiles of culture headspace gases for enrichment cultures that produced trimethylstibine, *Clostridium* spp. were selected for studies in monoseptic culture. The range of volatile compounds identified for mixed cultures was compared to known fermentation end-product profiles for individual clostridia species. Species were selected according to the following criteria;

- all fermentation products produced were detected in the GC-MS profile for headspace gases from urban wasteland soil enrichment cultures
- ability to grow in standard media
- ability to grow within temperature range of 25-35°C

Species consistent with these criteria are shown in Table 3.2.

Table 3.2 *Clostridium* spp. identified as being potentially present in cooked meat enrichment cultures of urban wasteland soil.

C.butyricum	*C.plagarum*	*C.carnis*
C.beijerinkii	*C.acetobutylicum*	*C.paraputrifium*
C.rubrum	*C.histolyticum*	*C.cochlearium*
C.pasteurianum	*C.septicum*	*C.lentoputrescens*
C.sporogenes	*C.tertium*	*C.tetanii*
C.botulinum	*C.spartagoforum*	

Of the range of clostridia species potentially present the following were chosen for screening for the ability to biomethylate antimony;

C.acetobutylicum (NCIMB 619)

C.butyricum (NCIMB 7423)

C.cochlearium (formerly *C.lentoputrescens*) (NCIMB 10629)

C.sporogenes (DMU culture collection).

C.acetobutylicum and *C.butyricum* were selected since the presence of ethanol, acetone, propanol, butanol and butanoic acid is indicative of butyric acid fermentation, and the identification of acetone is particularly indicative of the presence of *C.acetobutylicum* . *C.cochlearium* and *C.sporogenes* were chosen because they produce the full range of volatile fermentation products (excepting acetone) identified in the headspace of urban wasteland soil.

3.3.2 Trimethylstibine generation by undefined mixed cultures

Trimethylstibine was detected in the culture headspace of cooked meat medium using sample 2 (urban wasteland soil) as the inoculum. The frequency of detection was 50%. No other volatile forms of antimony, for example mono- or monomethylstibine, or stibine (SbH_3), were detected. A typical MS spectrum is shown in Figure 3.1.

When trimethylantimony dichloride standard was hydride generated and the resultant trimethylstibine trapped on Tenax traps and analysed by GC-MS, a detection limit of 0.35 μg trimethylstibine was obtained.

Trimethylstibine was also detected in the culture headspace of urban wasteland soil, cooked meat cultures not amended with inorganic antimony. No methylated antimony species were detected however by GC-AAS analysis of hydride generated soil inocula for this or any other soil samples. Neither were any methylantimony species detected in control cultures (non-inoculated media and media inoculated with sterile soil inocula). These data taken together indicate that the trimethylstibine arose through biological activity and not through reduction of pre-formed methylantimony species present in the soil or media. (The possibility of non-enzymatic transmethylation by other methylated species is discounted later section 3.3.3). The detection of trimethylstibine in cultures not supplied with potassium antimony tartrate suggests that the microbial population was capable of biomethylating inorganic antimony from very low levels already present in the soil or media. This enrichment condition (cooked meat medium) was the only one to be associated with antimony volatilisation. Volatile antimony species were not detected for either Fecult A or denitrifying (nitrate-reduction) enrichment conditions, nor for any of the other soil samples.

3.3.3 Methylantimony generation by monoseptic bacterial cultures

No volatile antimony species were detected in headspace gases of any monoseptic clostridia cultures. However GC-AAS analysis of hydride generated culture supernatants revealed the presence of involatile mono-, di-, and trimethylantimony species in varying proportions. The ratio of methylantimony species varied between clostridia species and time of incubation (Table 3.3). A typical GC-AAS spectra of

derivatised involatile antimony species present in clostridia culture supernatant is shown in Figure 3.2.

Table 3.3 Detection of methylantimony species in hydride generated culture broth of monoseptic clostridial cultures amended with 50 mg.l^{-1} antimony as potassium antimony tartrate.

Clostridium sp (redox potential)	Incubation period (days)	Methylantimony species in culture supernatant (ng.ml^{-1})		
		MeSb (st dev) n	Me_2Sb (st dev) n	Me_3Sb (st dev) n
C.acetobutylicum	0,2,7	nd	nd	nd
(-0.251 V)	14	9.0 (3.1) 7	4.4 (0.9) 2	0.9 (-)1
	28	9.5 (2.0) 3	3.5 (1.3) 3	8.3 (2.2) 5
C.butyricum	0	nd	nd	nd
(-0.401 V)	2	2.7 (1.1) 8	nd	nd
	7	4.1 (1.0) 8	nd	nd
	14	11.8 (3.3) 5	nd	3.7 (0.4) 3
	28	nd	nd	9.6 (1.7) 8
C.cochlearium	0,2,7	nd	nd	nd
(-0.321 V)	14	8.4 (2.6) 6	6.5 (2.1) 2	1.2 (0.2) 2
	28	nd	nd	3.7 (1.1) 7
C.sporogenes (-0.301 V)	0,2,7,14,28	nd	nd	nd
Isolates from enrichment culture				
Clostridium sp SI-1	0,2,7,14	nd	nd	nd
	28	0.66 (0.4) 7	0.76 (0.09) 2	nd
Clostridium sp SI-2	0,2,7,14	nd	nd	nd
	28	0.27 (0.12) 8	nd	nd

Eight replicate culture vials were analysed for each determination. In the calculation of means and standard deviations (st dev) only those culture vials (n) showing detectable levels of analyte were included. nd = not detected in any of the eight replicate culture incubations (<20 pg.ml^{-1}). All cultures were amended prior to incubation with 50 mg.l^{-1} potassium antimony tartrate. Redox potential measurements were taken after 28-days culture incubation.

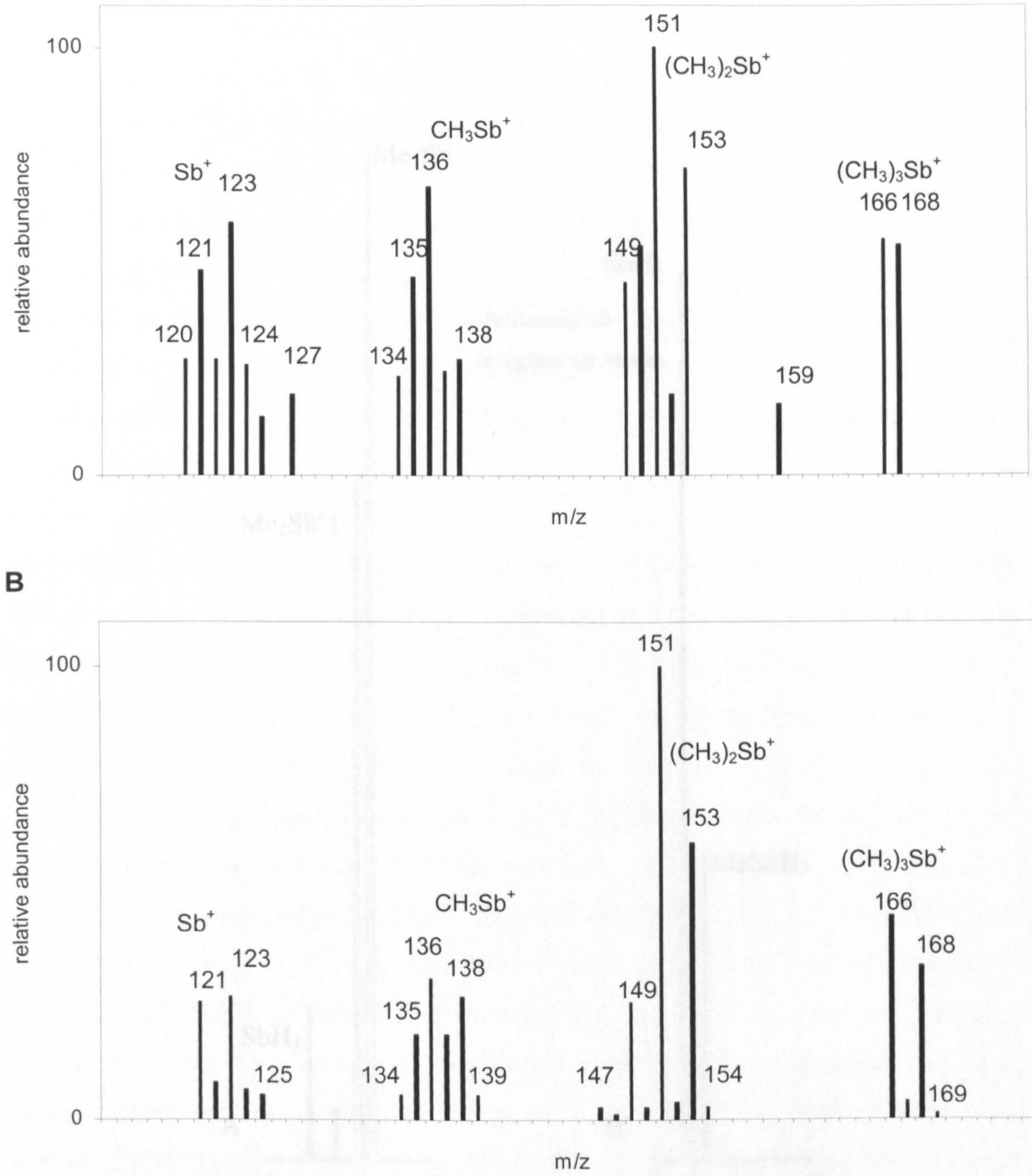


Figure 3.1 GC-MS identification of trimethylstibine. (A) Typical mass spectrum of trimethylstibine from culture headspace gases of urban wasteland soil enriched in cooked meat medium supplemented with 50 mg.l^{-1} antimony as potassium antimony tartate; (B) Reference mass spectrum of trimethylstibine from NIST library.

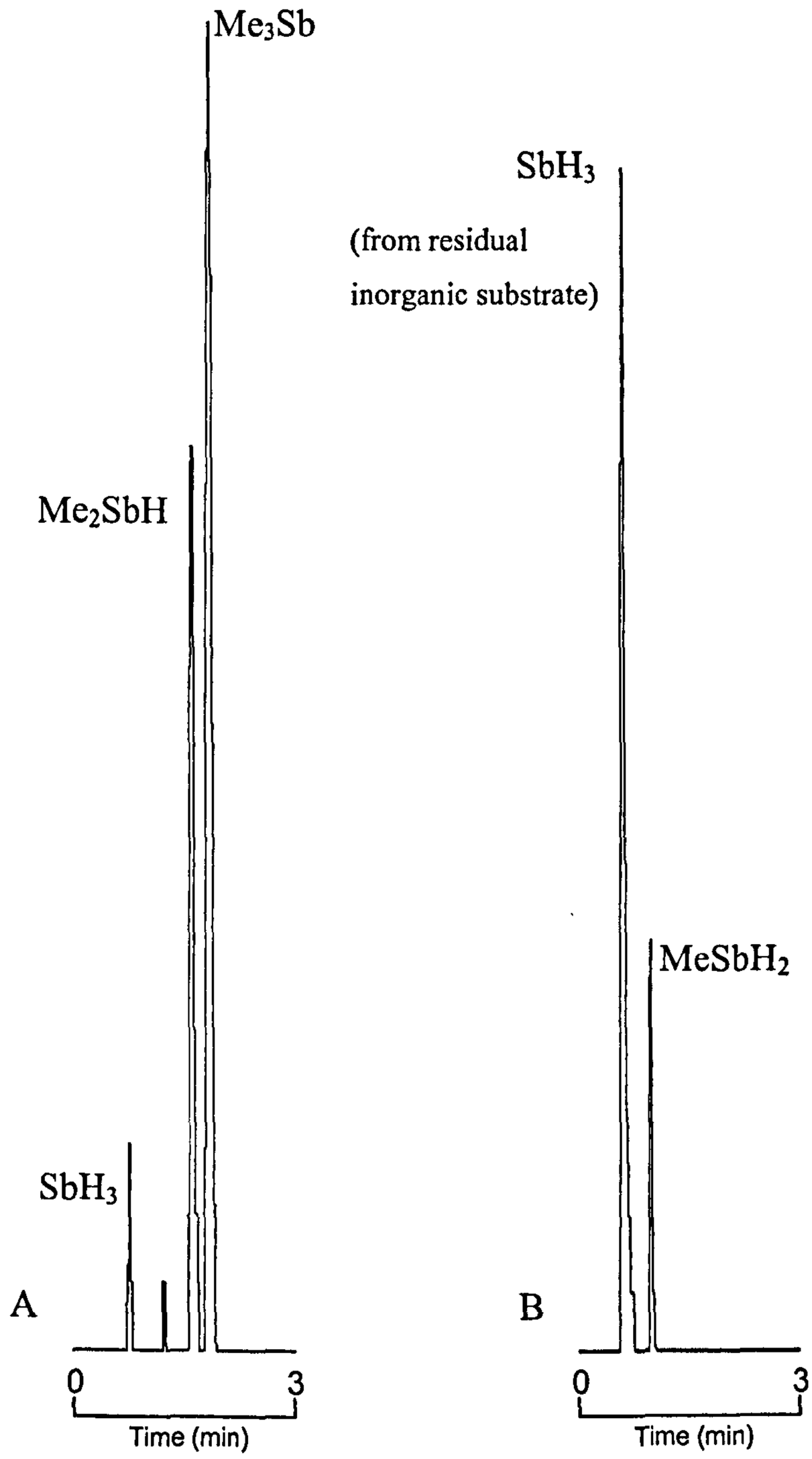


Figure 3.2 Typical GC-AAS chromatograms of (A) Volatile antimony compounds (standards) produced by reduction of trimethylantimony dichloride. Retention times are; stibine 0.79 min; monomethylstibine 1.33 min; monomethylstibine 1.71 min; trimethylstibine 1.94 min. (B) Volatile antimony species produced by hydride generation of monoseptic *Clostridium butyricum* culture supernatant (14-day incubation) amended with 50 mg.l^{-1} potassium antimony tartrate.

It should be noted that all data from GC-AAS analysis were adjusted to take into account any demethylation occurring during the hydride generation process, i.e. formation of mono-, or di- methylated antimony species from the demethylation of trimethylstibine. The degree of demethylation was calculated by reference to the demethylation observed upon hydride generation of trimethylantimony dichloride standard (the method used is detailed in Appendix I). The ratio of volatile antimony species was observed to be constant over the concentration range of trimethylantimony dichloride studied. It is not possible to state with certainty the identitiy of the antimony species found in the liquid phase, as hydride generation only provides information with regard to the oxidation state of a compound, i.e. with regard to alkyl metals, only the degree of alkylation can be described.

Monomethylantimony was detected in all culture incubations of soil isolates SI-1 and SI-2, at levels of up to 1.1 and 0.4 $ng.ml^{-1}$ respectively. Dimethylantimony was detected in only one culture, and trimethylstibine was not detected at any time. Monomethylantimony was the only methylated antimony species detected in *C.butyricum* cultures after 2 days (frequency of detection 100%). After 14 days, trimethylstibine was detected as the sole volatile antimony species in 38% of cultures, whilst monomethylstibine was detected as sole antimony species at a frequency of 62%. C.*cochlearium* and C.*acetobutylicum* displayed similar profiles of methylantimony production to *C.butyricum*, except that no methylated species were detected till after 14-days of incubation. In addition, monomethylstibine was detected in hydride generated culture broth for both species after 14-days, as with soil isolates SI-1 and SI-2, monomethylstibine was detected only in cultures that were positive for monomethylstibine too. These data suggest that each stage of biomethylation is induced sequentially, with di- and trimethylated species only being produced once a certain threshold limit of less methylated species is reached. Trimethylstibine was not detected in conjunction with other methylated antimony species in culture supernatants of any of the clostridial species tested, and no methylated species were detected in control cultures at any time. In addition, levels of trimethylstibine in clostridial cultures showed an apparent increase between 14 and 28-days of incubation. This suggests that a trimethylantimony species is the end product of the methylation pathway and indicates that this is an enzymatic process as opposed to transmethylation occurring as a result of changes in environmental conditions, brought about by microbial growth and

metabolism. No volatile antimony species were detected in hydride generated culture broth from *C.sporogenes* cultures at any time, indicating that this species does not possess a capability to methylate antimony. The percentage of supplied inorganic substrate that was methylated by clostridia was a tiny fraction of the whole, a maximum of 0.04% was observed (*C.acetobutylicum*, 28 days incubation). This would seem to suggest that this is not the primary resistance mechanism to antimony employed by these organisms, if indeed it is a resistance mechanism at all.

3.3.4 Redox potential of monoseptic cultures of anaerobic bacteria

No association between redox potential and percentage of inorganic antimony methylated was identified. However, it must be remembered that the sample population is too small for the results to have real significance to the microbial world in general. Redox potential were determined as; *C.butyricum* -0.401 V; *C.cochlearium* –0.321 V; *C.sporogenes* –0.301 V; and *C.acetobutylicum* –0.251 V. As it stands, this data suggests that reducing power alone is not the determining factor in whether an organism biomethylates antimony or not.

3.4 Discussion

Antimony biomethylation from inorganic antimony substrate has been demonstrated previously in enrichment cultures taken from methanogenic environments,[2, 3]. The identification of a biomethylating capability within the genus *Clostridium*, demonstrates that methanogens are not the sole biogenic methylating agents within a methanogenic environment. Indeed, methanogenesis is a multistage process in which formic acid fermentative bacteria and clostridia participate. The hydrogen and C-1 substrates required by methanogens are metabolised from complex organic polymers and other organic compounds by formic acid fermentors and clostridia during acetogenesis and fermentation.

Methylantimony species were detected by hydride generation-GC-AAS in the culture media of three of the four characterised *Clostridium* sp tested and of both clostridial isolates isolated from enrichment culture. Both the frequency of detection and mean level of monomethylstibine detected generally decreased with time of incubation, whereas those of trimethylstibine increased. For *C.butyricum*, monomethylstibine was present in the supernatant as the sole methylantimony species after only 2-days of incubation, but after 28-days trimethylstibine was the sole methylantimony species detected. The detection of trimethylstibine as sole methylantimony species at the end of the incubation period (28-days) for *C.cochlearium* and *C.butyricum* is consistent with trimethylstibine being the final product of antimony biomethylation by these bacteria, with mono- and monomethylstibine species appearing transiently in culture supernatants as intermediates of the antimony biomethylation pathway.

The Challenger mechanism [11] proposes a series of reduction and oxidative methylation steps for the methylation of arsenic from inorganic arsenic substrate to trimethylarsine oxide. It is generally accepted that antimony is biomethylated by a similar mechanism.[17] The detection of a trimethylantimony species as the sole hydride generated antimony species in culture broth from monoseptic culture incubations after 28-days, suggests that a trimethylated antimony compound is the metabolic end product of the biomethylation pathway in these organisms. This together with a time-dependant detection of the three methylated antimony species, i.e. mono → mono + di → tri, adds further weight to the acceptance of the Challenger model as the mechanism of microbial antimony methylation. In contrast to the fungus *S.brevicaulis*, for which it appears that the

transformation di → trimethylstibine is the rate determining step in the biomethylation pathway,[18] no accumulation of dimethylated species was observed. Whenever a trimethylated compound was detected in cultures, no other methylated species were identified as being present. The absence of methylated antimony species in control cultures (no biomass, or sterilised inoculum) indicates that the methylation observed is a biogenic process. The accumulation during the cultivation period of a trimethylated species as the sole hydride generated organoantimony species present indicates that the process is enzymatic in nature, and that the appearance of methylated species is not due solely to abiotic methylation by biogenically produced methylating agents, e.g. *S*-adenosylmethionine, or methylcobalamin, or abiotic methylating agents such as methyliodide or other organometals and metalloids. Furthermore, the accumulation of trimethylated antimony as sole involatile antimony species suggests that once induced, the transformation of di- to trimethylstibine is sufficiently up-regulated for supernatant levels of less methylated species to become negligible, i.e. the transformation of di- to trimethylstibine is not a rate limiting step.

Involatile mono-, di- and trimethylantimony compounds have been detected as products of antimony (III) biomethylation in supernatants from aerobic cultures of *S.brevicaulis* [17, 18] and *P.schwenitzii*.[19] Neither of these fungi produced significant quantities of monomethylstibine, which is contrary to the work reported on here; monomethylstibine was the sole product in early stages of incubation and in a later stage represented up to 76% of the methylantimony species formed (*C.butyricum* 14-days incubation). The data presented here on antimony biomethylation by *Clostridium* spp is consistent with the Challenger mechanism. However, contrary to antimony biomethylation by *S.brevicaulis*, monomethylstibine was never detected alongside trimethylstibine or as the principal methylantimony species. Abiotic oxidation of trimethylstibine [20] has been suggested to give rise to involatile methylantimony species in aerobic culture media of *S.brevicaulis*.[21] In the anaerobic culture media of clostridia, however, this is most unlikely to occur. Furthermore, detection of monomethylstibine as the sole methylantimony species in early stages of incubation does not support this notion (Table 3.3). A recent paper reported that monomethylstibine species are not among the oxidation products of trimethylstibine [22].

The maximum yield of methylantimony species from inorganic antimony substrate was 0.043% for *C.acetobutylicum* after 28-days incubation. By comparison, the maximum yield of methylantimony species produced from inorganic antimony substrate by *Clostridium collagenovorans* (based on Michalke *et al.*)[9] was ca. 710-fold lower. That only small proportions (< 0.05%) of the total inorganic antimony added to cultures were methylated suggests that antimony biomethylation by clostridia is probably fortuitous rather than a primary resistance mechanism for this element.

Michalke *et al.* [9] also reported on antimony biomethylation by three species of methanogenic archaea; the most productive species, *Methanobacterium formicicum*, had a maximum yield of methylantimony species from inorganic antimony substrate (0.064%) similar to that reported here for *C.acetobutylicum* (0.043%). The amounts of methylantimony species produced per unit volume of culture by the end of the incubation period for *M formicicum* and for C.*collagenovorans* were 54-fold and 5325-fold lower respectively,[9] compared to that for *C.acetobutylicum* at (21.3 $ng.ml^{-1}$). Differences in biomethylation capability of bacterial species and in cultivation conditions may partially account for these differences. However, whereas Michalke *et al.* assessed antimony biomethylation capability of *M.formicicum* and of *C.collagenovorans* based on measurement of volatile forms of methylantimony present in culture headspace gases that of *C.acetobutylicum* (reported here) was based on measurement of involatile methylantimony species present in the culture medium. It follows that accumulation of involatile forms of methylantimony – such as $CH_3Sb(OH)_2$, $(CH_3)_2SbOH$ and $(CH_3)_3SbO$ - in the culture medium of *C.collagenovorans* would have lead to an underestimation of the antimony biomethylation capability of this organism,[9] which could account for the apparent lower biomethylation capability of this organism when compared to *C.acetobutylicum*.

There are several reports of antimony biomethylation by undefined communities of bacteria grown under anaerobic conditions [1-3] and one by pure cultures of anaerobic bacteria.[9] Furthermore, the Challenger mechanism of arsenic biomethylation form inorganic arsenic substrate proposes a series of reductive methylation steps *via* trimethylarsine oxide to trimethylarsine. Antimony biomethylation capability may therefore be correlated to an ability to generate low redox potential environments. To test this using the characterised clostridia cultures, redox potential measurements were

taken at the end of the 28-day incubation period. No evidence for such a correlation was obtained. These data suggest that the formation of methylantimony compounds by mixed communities of bacteria under anaerobic conditions is determined by the presence of individual species with specific antimony biomethylating capability rather that by the overall environmental conditions set by mixed communities.

A variable detection frequency of volatile trimethylstibine in microbial cultures appears to be a feature of environmental analysis of this element, Jenkins *et al.* [3] reported a maximum detection frequency of 57%, whilst Gürleyük *et al.*[1] reported 50% detection frequency. This may be due to a number of factors such as variability in inoculum source or enrichment conditions between flasks, possible complexation to non-volatile, non-hydride generatable forms, or oxidation by residual O_2 present in flasks. The use of a soil inoculum was designed to increase inoculum homogeneity, but did not completely overcome the problem.

Two clostridia species were isolated from enrichment cultures that were positive for the production of trimethylstibine. Both were later identified as being capable of antimony biomethylation, suggesting that they must contribute at least to overall antimony biomethylation detected in urban wasteland soil cultures. This does not however preclude the possibility that other microbial species capable of biomethylation of the metalloid may also be present in enrichment culture incubations. The detection of methylantimony compounds in culture supernatants of pure clostridial isolates from soil enrichment cultures that were shown to generate trimethylstibine, suggests that mixed community functioning is not an obligate requirement for antimony biomethylation by undefined soil/sediment enrichment conditions that promote growth of clostridia.[2, 3]

The detection of trimethylstibine in the culture headspace gases of urban wasteland soil cultures, amended and non-amended with antimony, suggests that the enriched microbial population was capable of biomethylating inorganic antimony from low levels already present in the soil or media. Gürleyük *et al.* [1] noted a similar phenomenon in a culture incubation using automobile body shop soil as inoculum. No antimony was added to the culture, however trimethylstibine was detected by GC-fluorine induced chemiluminescence in culture headspace gases. These data suggest that if the biomethylation pathway is antimony induced, significant quantities of the metal are not

required and supports the notion that unlike arsenic biomethylation by fungi, antimony biomethylation by clostridia is probably a fortuitous rather than a primary mechanism for this element.

References for Chapter 3

1. H. Gürleyük, V. Van Fleet-Stalder, and T. G. Chasteen, Confirmation of the biomethylation of antimony compounds. *Applied Organometallic Chemistry* **11**, 471 (1997).
2. P. N. Gates, H. A. Harrop, J. B. Pridham, and B. Smethurst, Can microorganisms convert methylated antimony trioxide or potassium antimonyl tartrate to methylated stibines? *Science of the Total Environment* **205**, 215 (1997).
3. R. O. Jenkins, P. J. Craig, D. P. Miller, L. C. A. M. Stoop, N. Ostah, and T.-A. Morris, Antimony biomethylation by mixed cultures of micro-organisms under anaerobic conditions. *Applied Organometallic Chemistry* **12**, 449 (1998).
4. A. V. Hirner, J. Feldmann, R. Goguel, S. Rapsomanikis, R. Fischer, and M. O. Andreae, Volatile metal and metalloid species in gases from municipal waste deposits. *Applied Organometallic Chemistry* 8, 65 (1994).
5. J. Feldmann, R. Grümping, and A. V. Hirner, Determination of volatile metal and metalloid compounds in gases from domestic waste deposits with GC/ICP-MS. *Fresenius Journal of Analytical Chemistry* **350**, 228 (1994).
6. J. M. Brannon and W. H. Patrick Jnr., Fixation and mobilization of antimony in sediments. *Environmental Pollution (Series B)* **9**, 107 (1985).
7. A. V. Hirner, J. Feldmann, E. Krupp, R. Gruemping, R. Goguel, and W. R. Cullen, Metal(loid)organic compounds in geothermal gases and waters. *Organic Geochemistry* **29**, 1765 (1998).
8. J. Feldmann and A. V. Hirner, Occurence of volatile metal and metalloid species in landfill and sewage gases. *International Journal of Environmental and Analytical Chemistry* **60**, 339 (1995).
9. K. Michalke, E. B. Wickenheiser, M. Mehring, A. V. Hirner, and R. Hensel, Production of volatile derivatives of metal(loid)s by microflora involved in anaerobic digestion of sewage sludge. *Applied and Environmental Microbiology* **66**, 2791 (2000).
10. W. R. Cullen and K. J. Reimer, Arsenic speciation in the environment. *Chemical Reviews* **89**, 713 (1989).
11. F. Challenger, Biological methylation. *Chemical Reviews* **36**, 315 (1945).
12. S. Honschopp, N. Brunken, A. Nehrkorn, and H. J. Breunig, Isolation and characterisation of a new arsenic methylating bacterium from soil. *Microbiological Research* **151**, 37 (1996).
13. G. Gassmann and D. Glindemann, Phosphane (PH_3) in the biosphere. *Angewandte Chemie International Edition in English* **32**, 761 (1993).
14. T. L. Miller and M. J. Wolin, A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Applied Microbiology* **27**, 985 (1974).
15. P. J. Craig, S. N. Forster, R. O. Jenkins, and D. M. Miller, An analytical method for the detection of methylantimony species in environmental matrices: methylantimony levels in some UK plant material. *Analyst* **124**, 1243 (1999).
16. R. O. Jenkins, T.-A. Morris, P. J. Craig, A. Ritchie, and N. Ostah, Phosphine generation by mixed- and monoseptic cultures of anaerobic bacteria. *The Science of the Total Environment* **250**, 73 (2000).

17. P. Andrewes, W. R. Cullen, J. Feldmann, I. Koch, and E. Polishchuk, Methylantimony compound formation in the medium of *Scopulariopsis brevicaulis* cultures: $^{13}CD_3$-L-methionine as a source of the methyl group. *Applied Organometallic Chemistry* **13**, 681 (1999).

18. P. Andrewes, W. R. Cullen, J. Feldmann, I. Koch, P. E., and K. J. Reimer, The production of methylated organoantimony compounds by *Scopulariopsis brevicaulis*. *Applied Organometallic Chemistry* **12**, 827 (1998).

19. P. Andrewes, W. R. Cullen, E. Polishchuk, and K. J. Reimer, Antimony biomethylation by the wood rotting fungus *Phaeolus schweinitzii*. *Applied Organometallic Chemistry* **15**, 473 (2001).

20. G. E. Parris and F. E. Brinckman, Reactions which relate to environmental mobility of arsenic and antimony. II. Oxidation of trimethylarsine and trimethylstibine. *Environmental Science & Technology* **10**, 1128 (1976).

21. R. O. Jenkins, P. J. Craig, W. Goessler, D. Miller, N. Ostah, and K. J. Irgolic, Biomethylation of inorganic antimony compounds by an aerobic fungus: *Scopulariopsis brevicaulis*. *Environmental Science and Technology* **32**, 882 (1998).

22. P. J. Craig, S. A. Forster, R. O. Jenkins, G. Lawson, D. Miller, and N. Ostah, Use of mass spectroscopic techniques to elucidate the nature of the products of the oxidation of trimethylstibine in air. *Applied Organometallic Chemistry* **15**, 527 (2001).

4 BIOTRANSFORMATION OF ANTIMONY BY *CRYPTOCOCCUS HUMICOLUS*

4.1 Introduction

The biomethylation of arsenic is well established and has been extensively reviewed.[1-3] A range of microorganisms, including a number of fungi, can convert this metal to methylated arsenic. The tables below detail some of the fungi known to biomethylate arsenic.

Fungal biomethylation of arsenic to trimethylarsine was first elucidated by Challenger in 1932, [4] demonstrating that Gosio gas produced by *S.brevicaulis* from arsenic trioxide was trimethylarsine, and not diethylarsine or diethylarsine oxide, as had been suggested. The mechanism he proposed for the biomethylation of arsenic (and selenium and tellurium) involved a series of alternating reduction and methylation steps. The Challenger mechanism, as it has become known, has since been confirmed as the pathway for the biomethylation of arsenic in *S.brevicaulis* and *C.humicolus*.[5]

In contrast to the biomethylation of arsenic, reports of fungal biomethylation of antimony are very limited in number. Barnard [6] demonstrated a low level of antimony volatilisation by *P.notatum* from phenylstibonic acid and potassium antimoniate, and also demonstrated low levels of antimony volatilisation from dimethylstibonic acid by *P.notatum* and *S.brevicaulis*. Barnard suggested that the low level of antimony volatilisation, compared to that observed for arsenic, was related to the toxicity of the metal species; since antimony compounds were less toxic than the corresponding arsenic species, there was little requirement for the fungus to employ biomethylation as a resistance mechanism.

Since the work of Barnard, the fungi *S.brevicaulis* and *P.schweinitzii* have been demonstrated to possess the capability to produce trimethylstibine from inorganic antimony substrates. Both of these species are known arsenic biomethylators.[7, 8] Jenkins *et al.* [9]observed trimethylstibine production from potassium antimony tartrate by *S.brevicaulis*, and noted mobilisation of unknown volatile antimony species to distal nitric acid traps when culture incubations were supplied with potassium antimony tartrate, antimony trioxide, or antimony pentoxide. Andrewes *et al.* [10] also observed the production of low levels of trimethylstibine from potassium antimony tartrate by this organism.

Table 4.1 Fungal species known to methylate inorganic arsenic substrate.

Aspergillus fischeri	Thom and Raper (1932) [11]
Aspergillus glaucus	Bird *et al.* (1948)[12]
Aspergillus sydowi	Thom and Raper (1932) [11]
Cryptococcus humicolus	Cox and Alexander (1973) [13]
(formerly known as *Apiotrichum humicola)*	Cullen (1977) [14]
Gliocladium roseum	Cox and Alexander (1973) [13]
	Cullen (1977) [14]
Penicillium notatum	Challenger (1945) [7]
Penicillium sp.	Cox and Alexander (1973) [13]
Phaeolus schweinitzii	Barrett (1978) [8]
Rhodotorula rubra	Vidal and Vidal (1980) [15]
Saccharomyces ellipsoideus	Verona (1920) [16](disputed by Challenger)
Scopulariopsis brevicaulis	Challenger (1945) [7]
	Cullen (1977) [14]
Trichophyton rubrum	Zussman (1961) [17]
	(volatile arsine not definitively identified)

S.brevicaulis (Penicillium brevicaule), Aspergillus glaucus, A.virens **were demonstrated by Gosio [18]to evolve a garlic odour (characteristic of volatile arsine species) from arsenious oxide and various arsenical pigments; alkyl telluride, alkyl selenide and alkyl stibine compounds all display similar odour.**

Table 4.2 Fungal species known to transform organoarsenic substrates.

Species	Substrate	
Aspergillus niger	monomethylarsonic acid	Bird *et al.* (1948) [12]
	dimethylarsinic acid	
Monilia sitophilia Saccardo	dimethylarsinic acid	Pool (1912) [19]
Penicillium chrysogenum	monomethylarsonic acid	Challenger (1945) [7]
	dimethylarsinic acid	
Penicillium notatum	monomethylarsonic acid	Bird *et al.* (1948) [7, 12]
	dimethylarsinic acid	Challenger (1951) [20]
	chloroethylarsonic acid	
	phenylarsonic acid	

N.B. all species in Table 1 have also been demonstrated to transform various organoarsenic substrates.[1]

A number of authors have postulated that the capability to biomethylate arsenic (and antimony) is linked to resistance to the metalloid.[2, 21, 22] Indeed as already mentioned, postulated differences in toxicology of the two elements have been suggested as a cause for differences in biomethylation efficiencies.[6] The theory of biomethylation as a resistance mechanism arises out of the finding that organo forms of arsenic and selenium are less toxic than their inorganic counterparts.[2] Little is known about the cellular processing mechanisms for antimony in either prokaryotic or eukaryotic cells. Furthermore, it is often difficult to distinguish between toxicological effects elicited by antimony and those caused by arsenic, since the two elements are commonly associated in the natural environment and arsenic contamination of antimony chemicals has been reported.[23] Antimony is therefore generally regarded as possessing a similar toxicological profile to arsenic.[24-26]

The biosynthetic pathway first described by Challenger (1945) [7] is generally accepted as the mechanism for arsenic biomethylation. When the physico-chemical similarities of arsenic and antimony are considered, it is worth speculating upon the possibility of antimony biomethylation by a similar mechanism. The detection of involatile di- and trimethylated antimony species in aerobic incubations of *S.brevicaulis* and the intact transfer of the methyl group L-methionine-*methyl-d_3* to antimony [10, 27] supports this hypothesis.

Although fungal production of stibine has been suggested to be involved in the epidemiology of Sudden Infant Death Syndrome (SIDS),[28] the only reports of stibine (SbH_3) production by monoseptic microbial incubations are for *S.brevicaulis* (of ultra-trace levels),[10] and for the methanogen *Methanobacterium formicium*.[29] Reports of fungal production of arsine (AsH_3) are also scarce in the literature, with trimethylarsine being described as the sole species volatilised. It is perhaps worth considering however, that *bacterial* production of arsine is not unusual, and questions have been raised as to the suitability of certain columns for the separation of all volatile arsine and methylarsenic species.[30]

As yet, only one comparative report of antimony and arsenic biomethylation by the same microorganism has been described in the literature.[31] Given the similarity in physico-chemical properties of the two elements and the fact that they are commonly

found closely associated in real environmental samples, it is clearly important to establish not only the mechanistic details and extent to which antimony biomethylation can occur, but also the extent to which arsenic impacts upon this process.

This work has investigated the biotransformation of inorganic antimony species by the anamorphic basidomycetous yeast *C.humicolus*. This microorganism is a known biomethylator of arsenic [13] for which the Challenger mechanism of biomethylation using S-adenosylmethionine as methyl donor has been established.[5, 32]

4.2 Experimental

4.2.1 Preparation of *C.humicolus* cultures

C.humicolus was maintained by routine sub-culture on solidified YM-medium prepared as described by Yamada *et al.*[33]. YM medium was also used as a routine cultivation medium for this organism. Other media - nutrient broth, malt extract broth, R medium - were tested as cultivation media, however YM was observed to be the best in terms of reduced lag phase and amount of biomass obtained. Unless otherwise stated, YM medium was used for culture incubations. Media recipes and suppliers of proprietary media are shown in Appendix II.

Media were prepared such that the final volume occupied 40% of total flask volume, e.g. for a 500 ml flask 200 ml of medium was used. Culture inoculum was prepared from agar streak plates, incubated overnight at 28°C. Culture was swabbed off agar plates and resuspended in 10 ml aliquots of fresh liquid medium to produce a turbid cell suspension with an absorbance of 1 unit at $\lambda = 600$ nm. Inoculum was inoculated to culture medium at 0.5 ml inoculum per 100 ml. Culture inoculum was always prepared using the same medium type as required for culture incubations. Where no proprietary medium was available (e.g. malt extract agar – Oxoid), solid medium was prepared by the addition of 3% agar to liquid medium. Cultures were incubated at 28°C and 100 rpm in a Gallenkamp orbital incubator for periods of up to 28-days. Where necessary, control incubations were prepared omitting inoculation of biomass.

4.2.2 Additions of metal substrate to culture incubations

Antimony and arsenic were used as biotransformation substrates at various times. The standard concentration used was 50 mg (Sb or As).l^{-1}, although the metal concentration was varied for certain investigations in the range 1 – 5000 mg.l^{-1}. The metal was supplied in the three or five valency state and was dispensed from a concentrated sterile stock solution to sterile culture media immediately prior to inoculation.

Table 4.3 Metal additions to media.

Substrate (valency)	Formula	Concentration of stock solution ($g.l^{-1}$)	Supplier
Potassium antimony tartrate (III)	$KSbC_4H_2O_6$	10	Sigma-Aldrich (Poole, Dorset, UK)
Potassium hexahydroxyantimonate (V)	$KSb(OH)_6$	5	Sigma-Aldrich
Antimony trioxide (III)	Sb_2O_3	0.5	BDH(Lutterworth, Leic. UK)
Trimethylantimony oxide (III)	$Me_3Sb(OH)_2$	0.1	Synthesised in house (D.Miller, De Montfort University, Leicester, UK)
Trimethylantimony dichloride (III)	Me_3SbCl_2	0.1	Donated by W.Cullen, Uni. of British Columbia.
Sodium arsenate (V)	Na_2HAsO_4	10	Sigma-Aldrich
Sodium arsenite (III)	NaH_2AsO_3	10	Sigma-Aldrich
Disodium methylarsonate (V)	$Na_2MeAsO(OH)_2$	0.1	Synthesised in house (J.Edmonds, De Montfort University, Leicester, UK)
Dimethylarsinic acid (V)	$Me_2AsO(OH)_2$	0.1	Synthesised in house (J.Edmonds)

Arsenic impurity of potassium antimony tartrate and potassium hexahydroxyantimonate negligible.[34] Arsenic impurity of antimony trioxide = 0.4%.[34] Antimony impurity of sodium arsenate and sodium arsenite = negligible and 0.094% respectively.[34] Relative arsenic or antimony impurities of synthesised compounds was not assessed.

All metal solutions, with the exception of Sb_2O_3, were prepared by dissolving an appropriate amount of metal compound in sterile demineralised water. Sb_2O_3 solution was prepared by first dissolving the solid in HCl and then adjusting to pH 6. Solutions were stored in the dark at room temperature until required.

Control incubations were prepared as necessary without metal addition. Unless otherwise stated, after sampling, biomass was separated from supernatant by centrifugation, 10 minutes, 4000 rpm. Supernatants were subsequently passed through a basic alumina column (10 ml bed volume); methylantimony species were eluted from

the column using 0.1 mol.l^{-1} potassium acetate (pH 7.5) at a flow rate of 1 ml.min^{-1}. Supernatants were stored at –20°C until analysis.

4.2.3 Assessment of growth using potassium antimony tartrate or potassium tartrate as sole carbon source

R-medium was prepared as described previously, but with the omission of glucose. Sterile potassium antimony tartrate or potassium tartrate was added after autoclaving. Potassium tartrate was added such that the amount of carbon present was identical to that supplied by glucose, i.e. 15.7 g.l^{-1} potassium tartrate. Potassium antimony tartrate was added to achieve a final concentration of 50 mg.l^{-1} elemental metal and medium pH was adjusted to pH 5.5. (To add potassium antimony tartrate in equivalent carbon quantities to that of glucose would result in a final elemental metal concentration of 10,000 mg.l^{-1}.) Volumes (50 ml) of media were subsequently inoculated with 0.1 ml of a turbid *C.humicolus* cell suspension, and incubated at 100 rpm, 28°C. Culture absorbance (λ = 600 nm) was measured after 14-days and 21-days incubation.

4.2.4 Comparison of inhibitory effect of arsenic and antimony

YM media was prepared in double concentration and dispensed in 5 ml aliquots to universal vials. Aliquots of 10 g.l^{-1} potassium antimony tartrate (antimony III), sodium arsenite (arsenic III), or sodium arsenate (arsenic V) were added such that final concentrations of 10, 100, 500, 1000, and 5000 mg.l^{-1} metal in 10 ml total test volume were achieved. Test solutions of potassium hexahydroxyantimonate (antimony V) were prepared in an identical manner from a 5 g.l^{-1} solution. The maximum test concentration for antimony V was 2500 mg.l^{-1} antimony. All universal vials were inoculated with 0.1 ml overnight culture inoculum, resulting in an initial absorbance for test incubations of 0.22 units (λ = 600 nm). After 24 hours of incubation, absorbance of all test cultures was measured at 600 nm.

4.2.5 Assessment of association of antimony with biomass

To assess speciation and concentration of antimony associated with biomass, cells in early stationary phase were lysed. Culture broth of *C.humicolus* stationary phase culture (6-days incubation) was centrifuged, 10 minutes, 4000 rpm, and the cell pellet washed with fresh YM medium to remove non-associated antimony, and re-centrifuged. The cell pellet was finally re-suspended (to original absorbance) in YM fresh medium. Cell

lysis was achieved through a combination of physical disruption and enzymatic digestion; 2 ml of a 20 $mg.ml^{-1}$ lyticase solution (≈1000 units) (Aldrich) was added to 200 ml of the washed and re-suspended culture, and culture incubation continued for 1 hour, during which time a reduction in absorbance (λ= 600 nm) from 0.29 to 0.1 units was observed. Further breakage of cells was achieved through sonication of the partially lysed culture. β-mercaptoethanol (1 ml) (Aldrich) was added to soften cell walls prior to sonication, and polypropylene glycol (3 ml 10%, MW = 1080) (BDH) and 1 g sand (BDH) were added to prevent excessive sample foaming. Culture broth was sonicated at 16 microns for 8 x 1 minute intervals until $A_{600} \approx 0$ units. Culture broth was cooled on ice between sonications. Samples were taken pre- and post-lysis treatment, and a control culture (no enzyme addition, no sonication) was sampled. Following lysis, samples were centrifuged to remove particulate matter and prepared for hydride generation-GC-AAS analysis in the usual manner. Cell extracts were not fractionated, hence it is not possible to determine the distribution of intracellular and cell membrane/wall bound antimony.

4.2.6 Surface binding (adsorption) and uptake of antimony

YM medium (400 ml in each of three 1 litre flasks) was inoculated with *C.humicolus* and incubated for 5 days at 28°C and 100 rpm. Following incubation, the cultures were combined and the absorbance of the culture was adjusted to an absorbance of 4.4 units, equivalent to a concentration of 0.015 $g.ml^{-1}$ dry weight cells. Culture biomass was then concentrated ten-fold by centrifugation to a final volume of 120 ml. Concentrated biomass suspension (10 ml) was dispensed to 15 ml screw-capped, plastic centrifuge tubes. Antimony or arsenic stock solution (1 ml) was added such that a final concentration of 10 $mg.l^{-1}$ metal was achieved. To half the centrifuge tubes 1 ml of 0.26 $g.ml^{-1}$ solution of the respiratory inhibitor sodium azide was added, such that a final concentration of 0.22 $g.ml^{-1}$ was achieved. Sodium azide was used since it prevents ATP formation *via* inhibition of oxidative phosphorylation and will therefore inhibit active transport. A volume of 1 ml water was dispensed to the remaining flasks to achieve equivalent volume. Cell suspensions in centrifuge tubes were vortexed for 30 seconds and 1.3 ml aliquots removed for analysis (t = 0 sample). Tubes were then incubated on their sides in a rotary incubator at room temperature and 250 rpm. Aliquots (1.3 ml) were removed after 10, 15, 20, 30 and 60 minutes. A sample was additionally removed from "no azide" incubations after 18 hours. Upon removal of

samples from the test system, biomass and particulate matter were immediately separated from supernatant by microcentrifugation, 13000 rpm, 5 minutes. The resulting supernatant was stored at –20°C prior to inductively coupled plasma-atomic emission spectrometry (ICP-AES) analysis. A Perkin Elmer (Beaconsfield, Bucks., UK) Plasma 40 ICP-AES spectrometer was used for analysis. Samples were diluted ten-fold with Millipore filtered water and introduced into the plasma *via* a nebuliser at a flow rate of 0.3 $ml.min^{-1}$. Radio frequency of the R.F. coil was 40 MHz, and argon was used as plasma gas at a flow rate of 16 $l.min^{-1}$. Analysis of antimony was at $\lambda = 217.581$ nm, and of arsenic at $\lambda = 193.757$ nm. Spectral bandpass for either element was 0.5 nm.

4.2.7 Inhibition of protein synthesis

To establish whether antimony biomethylation is an induced enzymatic process, catalysed by constitutively expressed enzymes; or a result of simple transmethylation performed by abiotic methylating agents or biogenically produced compounds, protein synthesis in stationary phase cells (t = 3 days) was inhibited by the addition of cycloheximide (final culture concentration = 500 $\mu g.ml^{-1}$). Antimony was added to cultures at t = 0, or t = 3 days. The control incubation was amended with antimony from t = 0, and had no cycloheximide additions. Samples were removed from cultures at 3-day intervals for analysis by GC-AAS.

4.2.8 Labelling experiment

The ability of S-adenosylmethionine to serve as methyl donor in the biomethylation of antimony by *C.humicolus* was tested. L-methionine-*methyl-d_3* (0.27 g) was dissolved in sterile demineralised water (5 ml) and added to 400 ml YM medium giving a final concentration of 1.3 $mmol.l^{-1}$ Media was then inoculated and amended with 50 $mg.l^{-1}$ antimony as potassium antimony tartrate, and incubated as described before. After 19 days incubation, supernatant and biomass were separated by centrifugation and supernatant was subsequently passed through a basic alumina column as described before (section 2.1). Aliquots (200 ml) of supernatant were acidified to pH 2.5 and frozen until GC-MS analysis was performed. A control incubation (no L-methionine-*methyl-d_3*) was treated in an identical fashion.

4.2.9 Induction studies; effect of preconditioning cultures with arsenic or antimony

500 ml flasks of YM medium were prepared as standard (section 4.2.1). Prior to inoculation with *C.humicolus*, medium was amended with arsenic III as sodium arsenite or antimony III as potassium antimony tartrate as shown below;

1. no metal addition
2. 50 $mg.l^{-1}$ SbIII
3. 10 $mg.l^{-1}$ AsIII
4. 50 $mg.l^{-1}$ AsIII
5. 100 $mg.l^{-1}$AsIII

Cultures were incubated at 28°C, 100 rpm for 6 days at which point cells were separated from supernatant by centrifugation (10 min, 4000 rpm). Unbound residual metal associated with biomass was removed by washing the cell pellet with Ringer's solution (1/4 strength) (Oxoid). The pellet was resuspended in fresh Ringer's solution, vortexed and then recentrifuged. This procedure was repeated twice to ensure removal of unbound metal. The cell pellets were then resuspended in fresh YM medium amended with 50 $mg.l^{-1}$ antimony as potassium antimony tartrate and incubated at 28°C and 100 rpm for a further 16 days. Aliquots (20 ml) were removed every 3-4 days for GC-AAS analysis of involatile antimony species (section 4.2.12).

4.2.10 Competition studies; effect of co-incubation of arsenic and antimony on antimony biomethylation

YM medium containing 50 $mg.l^{-1}$ antimony as potassium antimony tartrate was amended with arsenic as sodium arsenite (AsIII) or as sodium arsenate (AsV) as described below, and then inoculated with *C.humicolus*

Arsenic concentration ($mg.l^{-1}$)	Ratio of As:Sb
0	no arsenic
50	1:1
10	1:5
5	1:10
250	5:1
500	10:1

Cultures were sampled after 14 days and analysed by GC-AAS for involatile antimony species (4.2.12). . Flasks were incubated at 28°C and at 100 rpm.

4.2.11 Remote trapping of culture headspace gases

C.humicolus cultures were prepared as already described (section 4.2.1) and amended with antimony III as potassium antimony tartrate, antimony V as potassium hexahydroxyantimonate, or arsenic V as sodium arsenate at 1000 $mg.l^{-1}$ final metal concentration. Culture flasks were fitted with Dreschel heads connected *via* PTFE tubing to a stoppered measuring cylinder containing 40 ml of concentrated nitric acid. Culture headspace gases were continuously purged through the nitric acid *via* a sintered glass sparger by a stream of sterile air (30 $ml.min^{-1}$). Cultures were incubated for a total of 13 days, after which time, total elemental metal concentrations in the nitric acid traps were determined. In addition, the nitric acid traps attached to certain culture flasks were replaced and sampled on a daily basis to obtain a volatilisation profile of the metal under investigation. Nitric acid trap samples were analysed by Dr W Gössler (Karl Franzens Universität, Graz, Austria) as described in section 3.2.1.

4.2.12 GC-AAS analysis of volatile arsenic and antimony species

C.humicolus cultures were prepared as described in section 4.2.1 and amended with potassium antimony tartrate, potassium hexahydroxyantimonate, sodium arsenite, or sodium arsenate at 50 $mg.l^{-1}$ final metal concentration. Cultures were incubated at 28°C, 100 rpm for 6 days, after which time they were concentrated tenfold by centrifugation (10 min, 4000 rpm) to a final volume of 60 ml. The resulting broth concentrate was transferred to a glass reaction vial and sealed with an aluminium crimp cap and PTFE lined butyl rubber septa. The sealed reaction vials were returned to the incubator and maintained at 28°C, 150 rpm for a further 16 days, the reaction vials were shaken to minimise deposition of biomass during the anaerobic incubation phase. After the incubation period was complete, culture headspace gases were transferred under a flow of helium (40 $ml.min^{-1}$) to a liquid nitrogen cooled column 50 cm x 4 mm i.d. glass wound with Ni-Cr resistance wire (80/20%) (10.04$\Omega.m^{-1}$)) packed with PT 10% OV101 on Chromosorb-W-HP (80/100mesh) (Alltech Associates, Deerfield, Illinois, USA). The transfer period was 10 minutes. After completion of the transfer period, the liquid nitrogen was removed and the column was heated electrothermally. Volatile species were eluted from the column according to their boiling point and analysed by a Perkin

Elmer (Beaconsfield, Bucks., UK) PE1300 atomic absorption spectrometer as described previously in section 3.2.6.

4.2.13 GC-AAS analysis of involatile antimony and arsenic species

Hydride generation-GC-AAS analysis was performed as described previously in section 3.2.6. Antimony species were analysed using an antimony specific lamp (λ = 217.6 nm). Volatile antimony standards were produced by hydride generation of potassium antimony tartrate and trimethylantimony oxide (retention times; stibine 0.79 min, monomethylstibine 1.33 min, dimethylstibine 1.71 min, trimethylstibine 1.94 min). Arsenic species were analysed using an arsenic specific lamp (λ = 193.7 nm). Volatile arsenic standards were produced by hydride generation of sodium arsenate, monomethylarsonic acid, dimethylarsinic acid and trimethylarsine oxide (retention times; arsine 0.55 min, monomethylarsine 1.08 min, dimethylarsine 1.39 min, trimethylarsine 1.58 min).

4.2.14 GC-MS analysis of volatile species

C.humicolus cultures were prepared as described before (section 4.2.1) and amended with 10 mg.l^{-1} and 50 mg.l^{-1} antimony as potassium antimony tartrate. Culture flasks were fitted with Dreschel heads connected *via* PTFE tubing to traps (6 cm x 5 mm) packed with 0.3 g Tenax®-TA (60/80 mesh). Culture headspace gases were continuously purged through the traps with air (30 ml.min^{-1}) for a period of 14 days. The experiment was repeated with liquid nitrogen cooled U-tubes packed with Tenax®-TA serving as headspace gas traps in effort to retain antimony species with a higher volatility than trimethylstibine, i.e. stibine, monomethylstibine and dimethylstibine. This was found to be problematic owing to the condensation of water within the trapping system and despite extensive insulation, fluctuations in the trap temperature as the liquid nitrogen volume reduced through evaporation. A continuous liquid nitrogen cooling system was impossible due to apparatus limitations. Culture headspace gases trapped on the Tenax®-TA were subsequently analysed for the presence of antimony species by GC-MS as described previously in section 3.2.5.

4.2.15 Solid-phase micro-extraction (SPME)-GC-MS analysis of volatile species

C.humicolus were prepared as described before (4.2.1) and incubated for 6 days at 28°C and at 100 rpm. Aliquots (31 ml) of culture were transferred to 35 ml reaction vials

containing a magnetic flea and sealed with a needle septum. Reaction vials were incubated anaerobically at 28°C and 100 rpm until SPME analysis was performed. Concentrated biomass cultures were also prepared for SPME analysis by concentrating six-day cultures ten-fold by centrifugation (4000 rpm, 10 minutes), and incubating as described above. SPME was performed as described previously in section 2.3.3.

4.2.16 GC-MS analysis of culture supernatants (including preparation of sample for analysis)

Samples were prepared for GC-MS analysis by hydride generation with trapping of volatiles to PT 10% OV101 on Chromosorb-W-HP (80/100mesh) chromatography support. 200 ml of thawed, acidified (pH 2.4 by addition of HCl) supernatant was deoxygenated with helium (60 $ml.min^{-1}$) for 10 minutes, and then hydride generated by the addition of 1 ml 8% $NaBH_4$. Helium flow was maintained throughout the hydride generation reaction time of 3minutes. After this time, the PT 10% OV101 on Chromosorb-W-HP (80/100mesh) trap was disconnected from the apparatus and the packing material removed to a liquid nitrogen cooled vial, by the application of a high-pressure nitrogen flow. The vial was immediately capped and returned to liquid nitrogen until analysis was performed.

GC-MS analysis was performed on a Varian 3300 gas chromatograph with a split-splitless injector and a Saturn 2000 mass spectrometer (ion trap) (Varian, Warrington, UK). Separations were performed using a 30 m x 0.25 mm film thickness 0.25 μm, wcot fused silica column (Varian). The inlet port and detector were maintained at 140°C and 50°C respectively, helium was used throughout as the carrier gas (2 $ml.min^{-1}$). The column was held at 35°C for 5 minutes and then ramped at a rate of 15°C.min^{-1} to a final temperature of 100°C and held at this temperature for a further 2 minutes. The oven was heated to 250°C for 10 minutes after each analysis to remove unwanted material from the column. The mass spectrometer was operated in electron impact mode monitoring positive ions. Scanning was over the range 40-200 mass units. Identification was based on the National Institute of Standards and Technology (NIST) library database of mass spectra. The retention time of trimethylstibine was determined by hydride generation of trimethylantimony dichloride to Tenax®-TA, retention time = 2.27 min. Prior to sample injection, the vial was removed from liquid nitrogen and warmed to facilitate the release of any methylated antimony species from the PT 10% OV101 on Chromosorb-W-HP

(80/100mesh) to the volatile phase. Up to 1000µl was injected to the GC injector port. The PT 10% OV101 on Chromosorb-W-HP (80/100mesh) was subsequently hydride generated *in situ* and up to 1000 µl aliquots analysed.

4.2.17 HPLC analysis of culture supernatants

Chromatographic separations were performed on culture supernatants using the method of Lintschinger *et al*..[35] An anion exchange Hamilton PRP-X100 column (150 x 4.1 mm, particle size 10 µm poly(styrene-divinylbenzene) trimethylammonium exchanger) was connected *via* a six-port Rheodyne Type 50 injection valve to an Alltech 425 HPLC pump. The analytical column was protected by a guard column packed with the same stationary phase. KOH (2 $mmol.l^{-1}$) was used as mobile phase at a flow rate of 1.5 $ml.min^{-1}$. Inorganic antimony III and V species were removed from samples prior to analysis by passage through a basic alumina column (as described previously section 2.1). Derivatisation and analysis of metal hydrides was performed as described previously in section 2.2.2. Retention times; trimethylantimony oxide 0.9 and 2.4 min (two peaks possibly as a result of degradation), potassium hexahydroxyantimonate 2.4 min.

4.3 Results

4.3.1 Utilisation of tartaric acid or potassium antimony tartrate as carbon source by *C.humicolus*

Cryptococcus sp. are known for their ability to utilise a wide range of carbon substrates for growth.[36, 37] The ability of *C.humicolus* to utilise tartrate as a carbon source was tested. Growth was monitored by measuring absorbance at 600 nm. After 2 weeks incubation some turbidity was observed in the tartaric acid incubations (A_{600} = 0.07 units), and after 3 weeks, incubations containing potassium antimony tartrate as carbon source also displayed slight turbidity (0.06 units). In contrast, growth in medium utilising glucose as carbon substrate was luxuriant (A_{600}= 4.3 units). At no point were high biomass levels observed in tartrate containing incubations indicating that whilst some low-level utilisation of tartaric acid and potassium antimony tartrate as carbon substrates may occur, they are not substrates of choice.

4.3.2 Resistance of *C.humicolus* to arsenic and antimony compounds

Antimony supplied in the III valency state as potassium antimony tartrate was observed to be more toxic, in terms of inhibition of growth, to *C.humicolus* than the antimony V compound potassium hexahydroxyantimonate (Figure 4.1). No effect on absorbance was noted at levels of up to 500 $mg.l^{-1}$ potassium hexahydroxyantimonate, in comparison to potassium antimony tartrate for which a loading of 10 $mg.l^{-1}$ metal resulted in a reduction in absorbance (at 600 nm) to 0.19 units (vs. no metal reference culture). A maximum antimony loading of 5000 $mg.l^{-1}$ antimony III resulted in severe retardation of growth, such that absorbance of the culture after 24 hours was only slightly higher than prior to the incubation test period. Morphological changes were observed after 16 days incubation in YM medium containing 1000 $mg.l^{-1}$ potassium antimony tartrate, growth was less luxuriant than the no-metal reference incubation, and cells displayed an anamorphic, non-mycelial form, with swollen and fragmented hyphae (Figure 4.2).

Arsenic V supplied as sodium arsenate displayed a similar inhibitory profile to potassium antimony tartrate. At loadings above 100 $mg.l^{-1}$ arsenate however, the inhibitory effect of the metal was even more marked. Indeed at 1000 $mg.l^{-1}$, culture absorbance was less than the initial absorbance of the test culture, indicating that in addition to growth inhibition, cell lysis occurred. A similar phenomenon was noted for

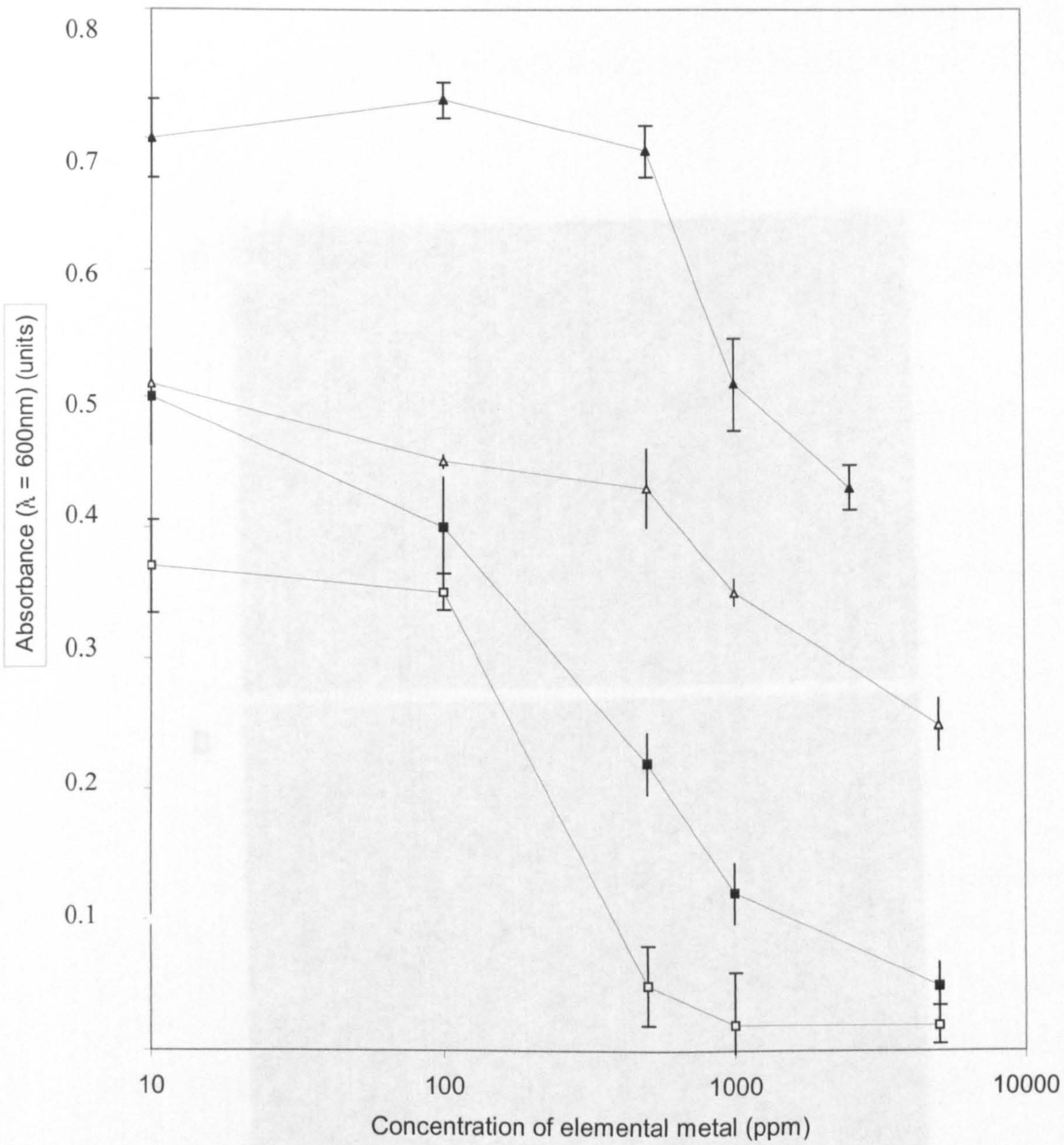
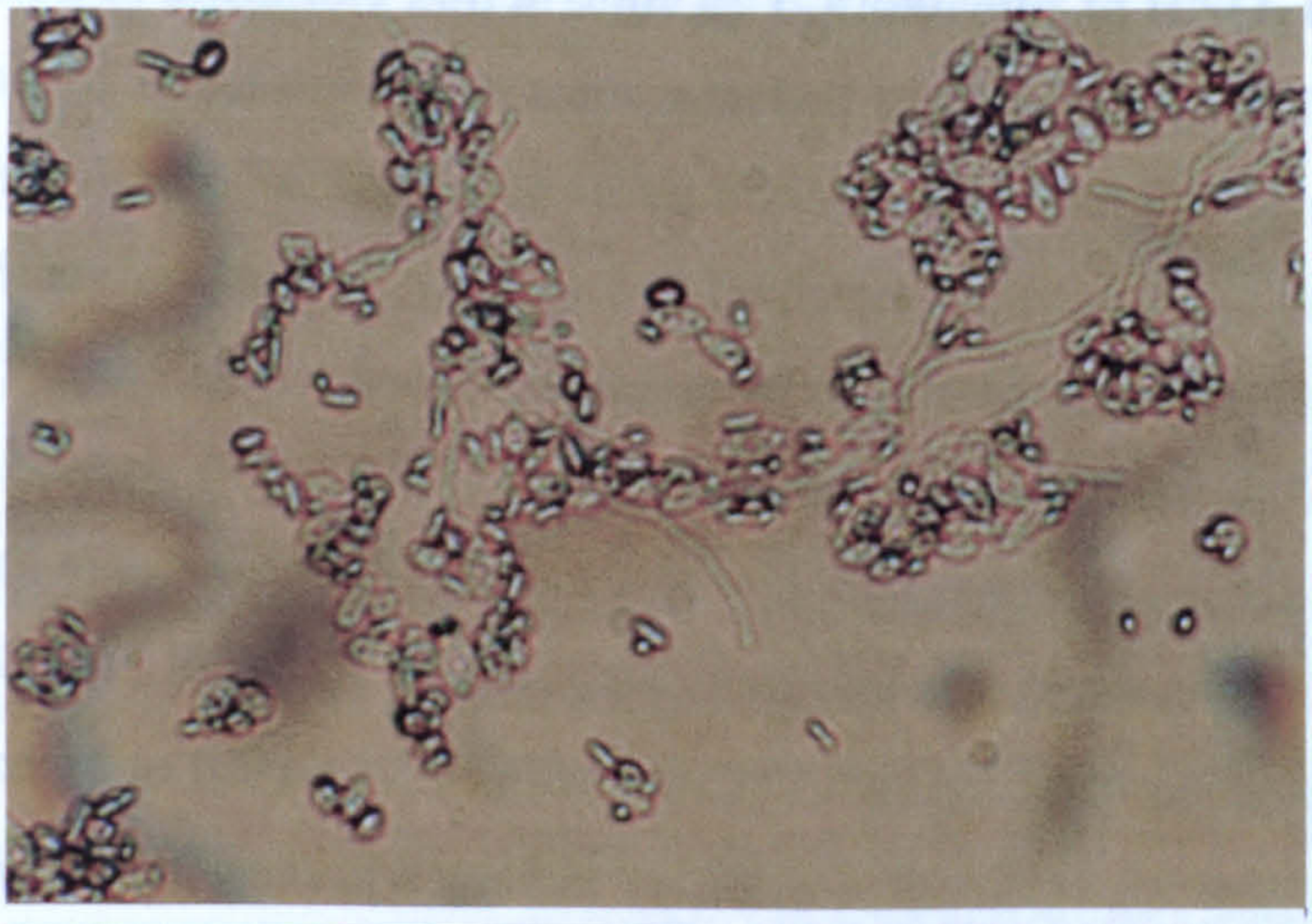


Figure 4.1 Effect of increasing antimony and arsenic load on growth of *C.humicolus*. Antimony was supplied as (△) potassium antimony tartrate or (▲) potassium hexahydroxyantimonate. Arsenic was supplied as (□) sodium arsenite or (■) sodium arsenate. 10 ml of YM media amended with the appropriate concentration of test metal was inoculated with 0.1 ml overnight culture inoculum and incubated for 24 hours, after which time absorbance (λ = 600 nm) was recorded as a measure of biomass. A_{600} of no metal reference culture after 24 hours = 0.7 units.

A



B

Figure 4.2 Morphological changes observed in *C.humicolus* when grown in media containing elevated levels of antimony III. x400 magnification. (A) *C.humicolus* in YM media, 16 days incubation. (B) *C.humicolus* in YM media + 1000 mg.l^{-1} potassium antimony tartrate, 16 days incubation.

the arsenic III, supplied as sodium arsenite, incubations at 500 $mg.l^{-1}$. Yeast and fungi often exhibit such high tolerance to the inhibitory effects of metal ions, for example, the yeast *Saccharomyces cerevisiae* can tolerate up to 150 $mg.l^{-1}$ sodium arsenite, and growth is not completely inhibited until levels are increased to 700 $mg.l^{-1}$ sodium arsenite.[38]

4.3.3 Mobilisation of antimony and arsenic by *C.humicolus* to HNO_3 traps

ICP-MS analysis of the distal HNO_3 traps attached to *C.humicolus* cultures indicated that some volatilisation of antimony occurred during cultivation (Table 4.4). Since any volatilised antimony and arsenic species will be destroyed (e.g. C-Sb and C-As bonds will be cleaved) in the HNO_3 traps, speciation of mobilised antimony and arsenic was not possible. Antimony in the SbIII state as potassium antimony tartrate was volatilised more readily than antimony V as potassium hexahydroxyantimonate. The mean amount of antimony mobilised to HNO_3 traps per day from inorganic antimony III substrate was 23.4 $\mu g.g^{-1}$compared to 0.2 $\mu g.g^{-1}$from antimony V. Mobilisation of arsenic was higher than mobilisation of antimony from either valency. A factor of 10^3 was noted between antimony III and arsenic V incubations and 10^4 between antimony V and arsenic V incubations (arsenic III was not tested). Jenkins *et al.* [9]reported a similar finding of higher mobilisation efficiency from antimony III compounds for the fungus *S.brevicaulis* using the same methodology. Low levels of passive mobilisation of metal from incubations containing no biomass was found. Total amounts present in these HNO_3 traps after 13-days incubation was up to 0.6 µg (no biomass + potassium antimony tartrate). This passive metal mobilisation could not account for the total amounts detected in incubations containing biomass and indicates that biogenic activity is responsible for the higher mobilisation levels observed in these cultures.

Volatilisation of various arsenic compounds to trimethylarsine by *C.humicolus* has been described by Cox and Alexander [13] and confirmed by Cullen *et al.*.[14] Trimethylarsine was the only volatile arsine species detected by either group, no arsine or other methylated species were identified. It is possible to conclude therefore that the total arsenic volatilisation observed here may comprise trimethylarsine alone. The most productive stage of arsenic volatilisation was during the early log phase of growth (Figure 4.3). The rate of volatilisation slowed during the late log and linear phases of

Table 4.4 ICP-MS analysis of antimony and arsenic mobilised to distal HNO_3 traps during cultivation of *C.humicolus* in the presence of inorganic metal substrate at 1000 mg.l^{-1}.

Incubation	Total amount of metal mobilised to trap in 13 days		Mean amount of metal mobilised per g dry weight biomass per day	
	Sb (µg)	As (mg)	Sb (µg.g^{-1}per day)	As (mg.g^{-1}per day)
C.humicolus + potassium antimony tartrate	3.5 (1.6)	na	23.4 (6.7)	na
No biomass + potassium antimony tartrate	0.6 (0.3)	na	na	na
C.humicolus + potassium hexahydroxyantimonate	0.6 (0.1)	na	0.2 (0.0)	na
No biomass + potassium hexahydroxyantimonate	0.4 (0.1)	na	na	na
C.humicolus + sodium arsenate	na	1.9 (0.2)	na	11.2 (0.3)
No biomass + sodium arsenate	na	0.2 (0.1)	na	na

Figure in parentheses are standard deviations based on three replicate culture incubations; na = not analysed; up to = 0.59 (0.28) µg over 13 days mobilised in the absence of biomass; total background antimony and arsenic in 40 ml HNO_3 trap was 24 ng and 40 ng respectively.

growth, but appeared to increase again as the death phase progressed (indicated by a concurrent decrease in absorbance). This biphasic volatilisation pattern may suggest that more than one mechanism is responsible for volatilisation of arsenic, possibly release of volatile arsenic species through autolysis.

4.3.4 GC-AAS analysis of volatile antimony and arsenic species

Volatile antimony species were detected in the headspace gases from incubation vials (transferred under helium flow to a liquid nitrogen cooled column) containing *C.humicolus* biomass (10-fold concentrated) and inorganic antimony.

Stibine and trimethylstibine were detected in cultures supplied with antimony V as potassium hexahydroxyantimonate. Stibine was the predominant species with up to 7.3 ng stibine and 3.11 ng trimethylstibine per g dry weight biomass being detected (Table 4.5). In contrast, trimethylstibine was the sole volatile antimony species detected in headspace gases from culture incubations supplied with antimony III as potassium antimony tartrate (Figure 4.4).

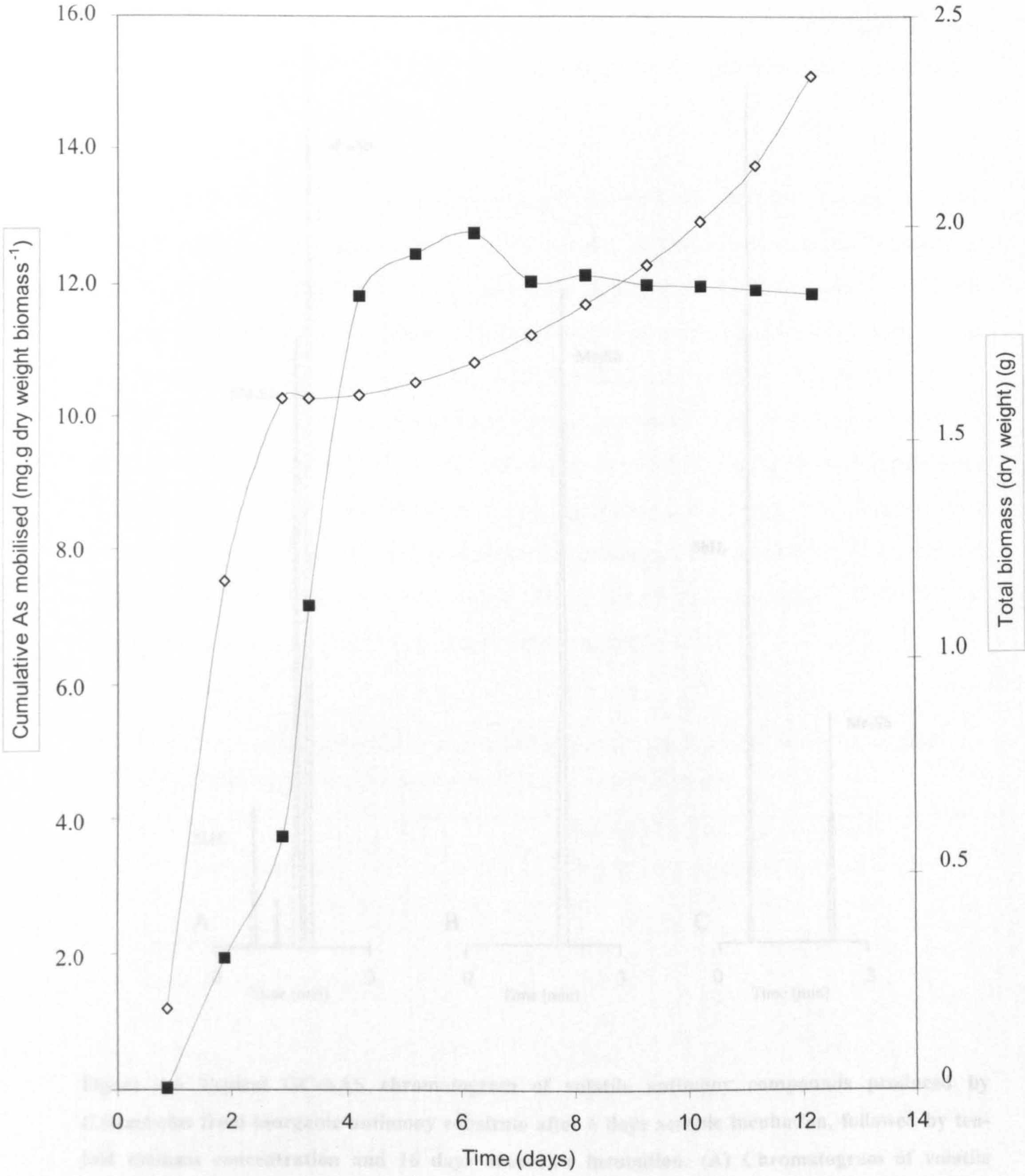


Figure 4.3 Mobilisation of arsenic to distal HNO_3 traps during the cultivation cycle of *C.humicolus*. The culture medium contained arsenic at 1000 $mg.l^{-1}$ supplied as sodium arsenate. HNO_3 traps were replaced at intervals and total arsenic concentration therein was determined by ICP-MS. (◇) Cumulative As mobilised ($mg.ml^{-1}$). (■) total biomass (dry weight) (g).

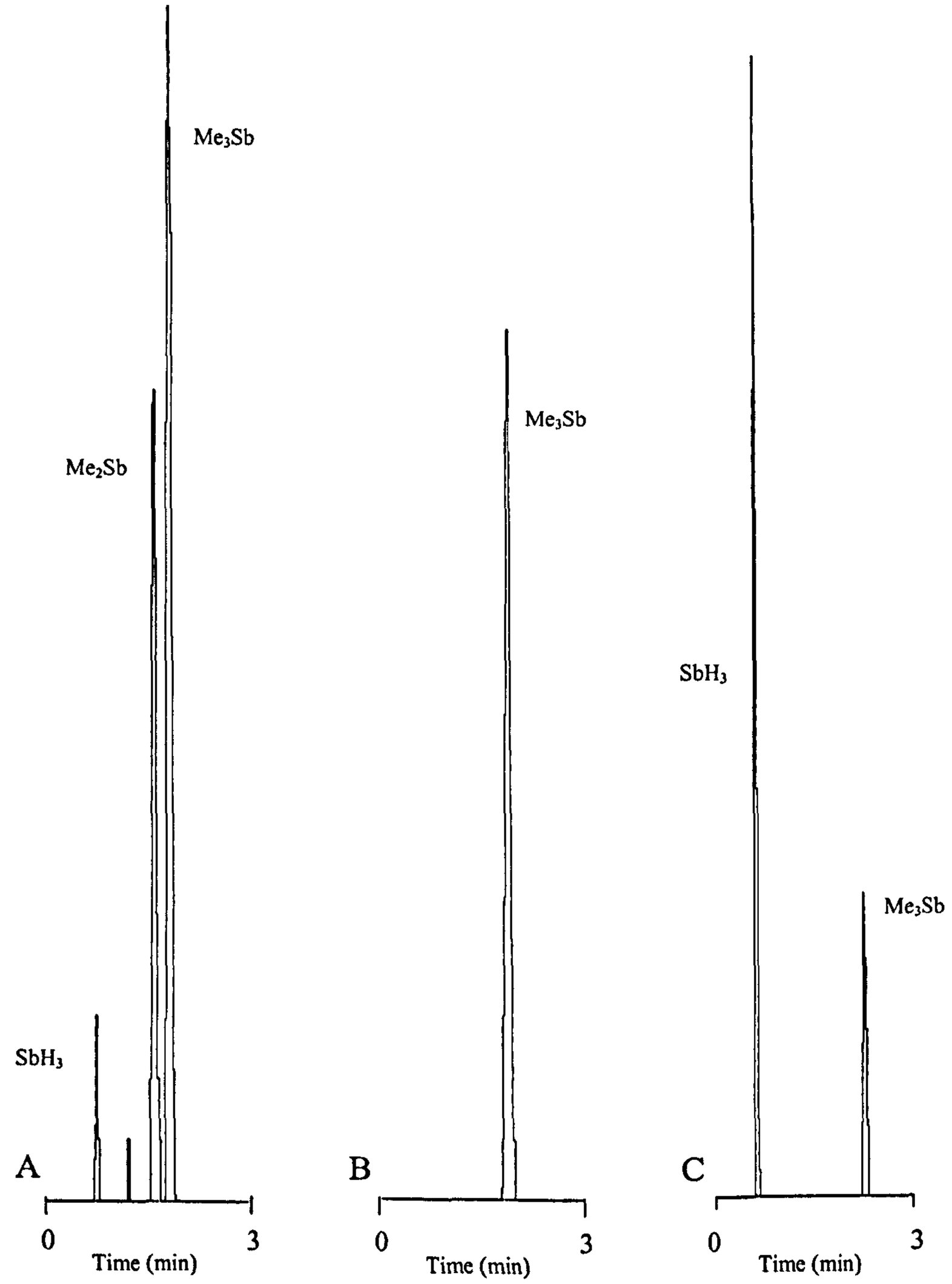


Figure 4.4 Typical GC-AAS chromatogram of volatile antimony compounds produced by *C.humicolus* from inorganic antimony substrate after 6 days aerobic incubation, followed by ten-fold biomass concentration and 16 days anaerobic incubation. (A) Chromatogram of volatile antimony (standards) produced by reduction of trimethylantimony dichloride. Retention times are stibine 0.79 min, monomethylstibine 1.33 min, dimethylstibine 1.71 min, trimethylstibine 1.94 min. (B) Culture supplied with 50 mg.l^{-1} potassium antimony tartrate. (C) Culture supplied with 50 mg.l^{-1} potassium hexahydroxyantimonate.

Trimethylstibine production was higher in incubations supplied with antimony III compared to those supplied with antimony V. Up to 22.7 ng per g dry weight biomass was observed, nearly ten-fold higher than trimethylstibine production from antimony V substrate.

GC-AAS analysis of culture headspace gases of *C.humicolus* biomass incubations (ten-fold concentrated) amended with inorganic arsenic substrate revealed the presence of arsine and trimethylarsine. Both species were detected whether the initial inorganic substrate supplied was in the III or V valency state. No other volatile arsenic species e.g. monomethylarsine or dimethylarsine, were detected at any time. Again the reduced hydride (i.e. arsine) was the predominant species present in incubations supplied with V valency substrate, and trimethylarsine was higher in incubations supplied with inorganic III substrate, i.e. sodium arsenite). Up to 23.8 ng arsine, and 4.8 ng trimethylarsine per g dry weight biomass was detected in incubations amended with arsenic V. This compares with incubations amended with arsenic III, in which 5.5 ng arsine, and 14.2 ng trimethylarsine per g dry weight biomass was detected.

Table 4.5 GC-AAS analysis of volatile arsenic and antimony species present in headspace gases from *C.humicolus* incubations supplied with inorganic metal substrate.

Metal substrate	Amount of volatile species detected (ng Sb per g dry weight biomass)	
	MH_3	Me_3M
Potassium antimony tartrate	nd	22.7 (3.5)
Potassium hexahydroxyantimonate	7.3 (2.1)	3.1 (1.6)
Sodium arsenite	5.5 (2.8)	14.2 (3.5)
Sodium arsenate	23.8 (4.0)	4.8 (2.7)

nd = not detected (< 1ng absolute); figure in parentheses are standard deviations based on three replicate culture incubations; M = metal being As for incubations supplied with inorganic arsenic and Sb for those supplied with antimony.

Volatilisation from inorganic arsenic substrates using this methodology was of similar quantity to the volatilisation observed in identical incubations supplied instead with antimony III as biotransformation substrate. This is unexpected as it is generally accepted that biovolatilisation of arsenic is more favourable than that of antimony.[27] Data obtained from the fully aerobic HNO_3 trapping experiments (section 4.3.3) are consistent with this.

4.3.5 SPME-GC-MS analysis of volatile antimony species produced by *C.humicolus*

No clear detection of volatile antimony species was obtained from SPME-GC-MS analysis of culture headspace gases from non-concentrated cultures. A small amount of stibine was noted after 19 hours of anaerobic incubation following 6-days aerobic incubation. However, amounts were extremely low and were not detected during subsequent analyses after 91 hours or 280 hours of anaerobic incubation. Analysis of the headspace gases from *concentrated-biomass* antimony V amended incubations after 18 days anaerobic incubation revealed the presence of stibine, dimethylstibine and trimethylstibine (Figure 4.5). Stibine was the predominant antimony species detected, with amounts per g (dry weight) biomass being; stibine, 110.6 $ng.g^{-1}$, dimethylstibine, 5.1 $ng.g^{-1}$; trimethylantimony, 11.1 $ng.g^{-1}$. In contrast, trimethylstibine was the sole volatile antimony species detected in the headspace of concentrated-biomass incubations amended with antimony III. The total amount detected was of the order 22.1 $ng.g^{-1}$. No volatile antimony species were detected in any of the control incubations indicating that the volatile antimony species detected arose as a result of biological activity.

4.3.6 Surface binding and uptake of arsenic species by *C.humicolus*

When arsenic was supplied in the three valency state the association (surface binding and uptake) of metal with biomass was much higher than that observed for the V valency state. Up to 31.23 μg arsenic was associated per g biomass (dry weight) over a 1-hour period for sodium arsenite compared to 19.98 $\mu g.g^{-1}$ for sodium arsenate. (Table 4.6). Lerman *et al.* [39]noted a similar observation for the uptake of arsenic by hepatocytes and suggested that the differences in uptake between the two valencies was due to differences in ionisation at physiological pH for the two forms. Arsenic III (pKa = 9.23) is uncharged and could therefore enter the cell *via* diffusion (passive or facilitated), whereas arsenic V (pKa = 2.20) is charged. The supposition that cellular uptake of sodium arsenite is a diffusion process is further supported by the observation that upon incubation with the respiratory inhibitor sodium azide, no change in the rate of association of metal with biomass was observed. The decrease in the rate of metal-biomass association with time indicates that the uptake of sodium arsenite occurs by facilitated not passive diffusion.

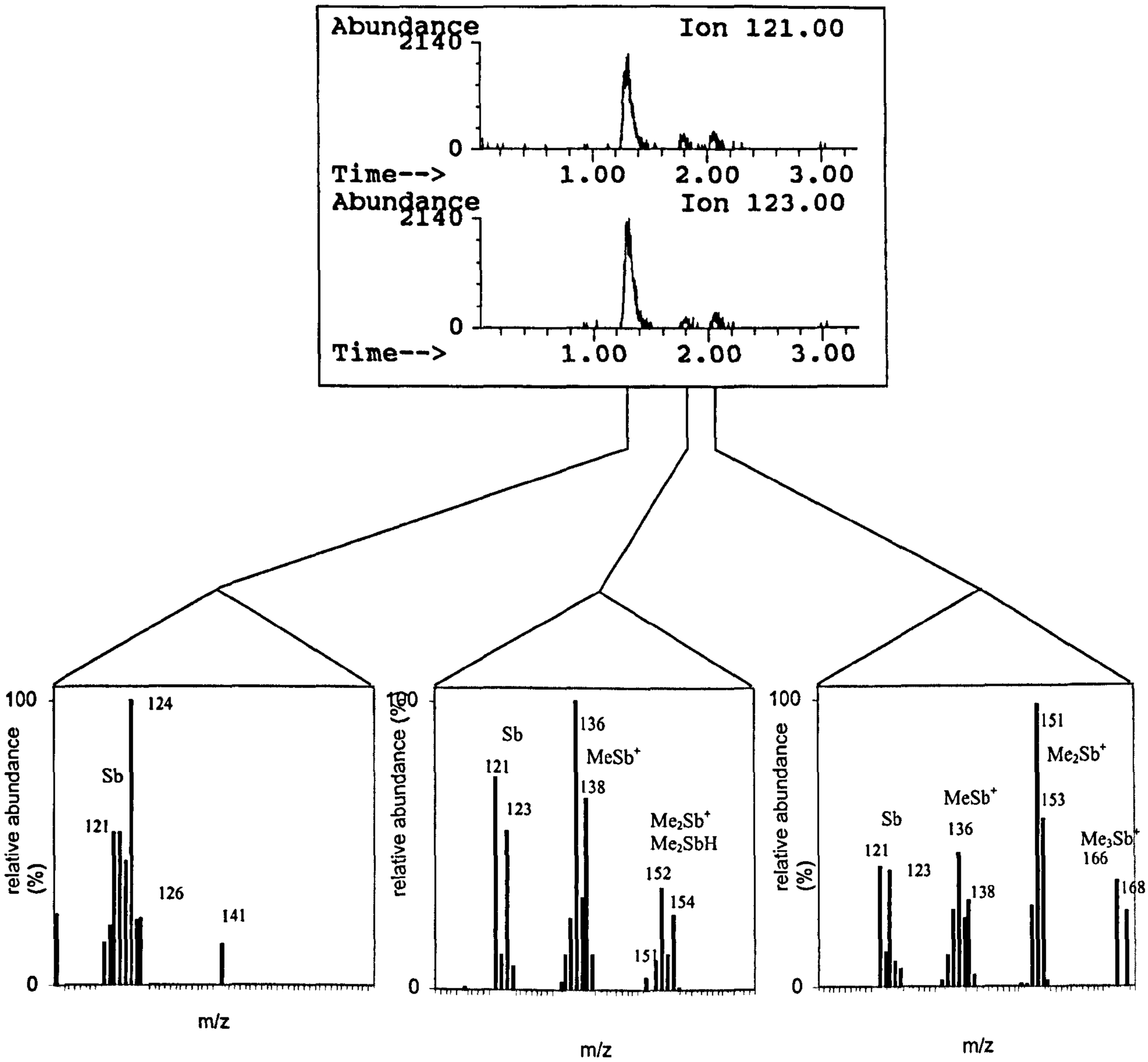


Figure 4.5 Mass/ion chromatogram and fragmentation patterns of (from left to right) stibine, dimethylstibine and trimethylstibine obtained by solid-phase micro-extraction of headspace of *C.humicolus* cultures (ten-fold biomass concentrated) supplied with potassium hexahydroxyantimonate.

Incubation of *C.humicolus* biomass with sodium arsenate and sodium azide however, resulted in a 92% reduction in the rate of metal-biomass association, indicating that the uptake mechanism of the V valency state is an active process dependent upon ATP. No large differences in initial rates of metal-biomass association for either valency were noted when sodium azide was included in the incubation, since surface binding - which would be the primary mechanism of association during this period - is a passive process. Differences in initial rates of metal-biomass association were however noted between the two valency states, probably because of differences in surface binding to ligand groups. For example, arsenic III is known to interact strongly with sulfhydryl groups. The initial rate of metal-biomass association for sodium arsenite was 2.71 $\mu g.g^{-1}$ (dry weight). min^{-1} compared to 0.92 $\mu g.g^{-1}.min^{-1}$ for sodium arsenate. Trimethylarsenic oxide also associated with *C.humicolus* biomass, however uptake of the compound was a passive diffusion process since inclusion of sodium azide in the incubation mixture did not affect the uptake rate of trimethylarsenic oxide, and metal-biomass association was linear with time once initial metal-biomass association was complete.

Table 4.6 Association of arsenic compounds with *C.humicolus* biomass.

A

Compound	Initial rate of metal-biomass association t = 0 - 5 min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 5 - 60 min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 1 - 18 h ($\mu g.g^{-1}.min^{-1}$)
Sodium arsenite	2.71	0.23	0.05
Sodium arsenate	0.92	0.11	0.01
Trimethylarsenic oxide	1.51	0.09	0.09

B

Compound	Initial rate of metal-biomass association t = 0 - 5min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 5 - 60min ($\mu g.g^{-1}.min^{-1}$)	Reduction in metal-biomass association rate when incubated with sodium azide (%)
Sodium arsenite	2.61	0.24	0
Sodium arsenate	0.80	0.01	92
Trimethylarsenic oxide	1.44	0.09	0

Reaction mixture in YM media (volume (10 ml) contained (A) *C.humicolus* 1.5 g dry weight biomass; arsenic 100 μg; and (B) *C.humicolus* 1.5 g dry weight biomass; arsenic 100 μg; sodium azide 26 μg. Final biomass association after 18-hours incubation (no sodium azide); sodium arsenite 77.17 (1.77) $\mu g.g^{-1}$ (dry weight biomass); sodium arsenate 20.85 (2.31)) $\mu g.g^{-1}$; trimethylarsenic oxide 61.33 (3.79)) $\mu g.g^{-1}$. Figure in parentheses represent standard deviation of three replicate incubations.

4.3.7 Surface binding and uptake of antimony species by *C.humicolus*

As with arsenic, the association (surface binding and uptake) of the III valency state of the metal with *C.humicolus* biomass was much higher than that observed for the V valency state. When antimony was supplied in the III valency state as either potassium antimony tartrate or antimony trioxide up to 12.6 μg antimony was associated per g biomass (dry weight) after 1-hour incubation. (Table 4.7). The association of antimony with biomass when supplied in the III valency state was greater (6-fold) than when supplied as antimony V, i.e. as potassium hexahydroxyantimonate. Up to 2.3 $\mu g.g^{-1}$ metal-biomass association was noted when biomass was incubated with antimony V for a 1-hour period. At physiological pH, antimony V (pKa = 2.7) is ionised, whereas antimony III is uncharged (pKa = 11.6) and can therefore enter the cell *via* passive or facilitated diffusion. Little difference in the rates of metal-biomass association was observed between antimony trioxide incubations including and omitting sodium azide. This indicates that the cellular uptake of antimony trioxide is a diffusion process. That the rate of antimony trioxide association with biomass in both sodium azide and non-sodium azide incubations decreased with time indicates that this is a facilitated diffusion process. For potassium hexahydroxyantimonate, an 82% reduction (from 0.017 $\mu g.g^{-1}.min^{-1}$ to 0.003 $\mu g.g^{-1}.min^{-1}$) in the rate of metal association with biomass was noted, demonstrating that the uptake of this compound by *C.humicolus* is an active process dependant upon ATP. Interestingly the rate of uptake of potassium antimony tartrate (antimony III) was also inhibited by the presence of sodium azide, a reduction of 36%

(from 0.14 $\mu g.g^{-1}.min^{-1}$ to 0.09. $\mu g.g^{-1}.min^{-1}$) was observed. This indicates that the uptake of potassium antimony tartrate by *C.humicolus* is at least in part dependent upon ATP. A possible explanation may be that some dissociation of potassium antimony tartrate occurs in solution resulting in equilibrium of Sb^{3+} ions, which would enter the cell *via* diffusion, and tartaric acid and potassium antimony tartrate. Uptake of the tartrate would occur *via* active transport since the compound is charged at physiological pH. (pKa of potassium tartrate is 2.98) and uptake would therefore be inhibited by the presence of sodium azide. In contrast to trimethylarsenic oxide, trimethylantimony oxide did not associate with *C.humicolus* biomass at any time.

Table 4.7 Association of antimony compounds with *C.humicolus* biomass.

A

Compound	Initial rate of metal-biomass association t = 0 - 5 min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 5 - 60 min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 1 - 18 h ($\mu g.g^{-1}.min^{-1}$)
Potassium antimony tartrate	0.70	0.14	0.03
Antimony trioxide	0.82	0.09	0.02
Potassium hexahydroxyantimonate	0.19	0.02	0.004
Trimethylantimony oxide	0.0	0.0	0.0

B

Compound	Initial rate of metal-biomass association t = 0 - 5 min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 5 - 60 min ($\mu g.g^{-1}.min^{-1}$)	Reduction in metal-biomass association rate when incubated with sodium azide (%)
Potassium antimony tartrate	0.77	0.09	36
Antimony trioxide	0.88	0.09	0
Potassium hexahydroxyantimonate	0.21	0.003	82
Trimethylantimony oxide	0.0	0.0	0

Reaction mixture was as described for Table 4.6, antimony supplied at 130 µg. Final biomass association after 18-hours incubation (no sodium azide); potassium antimony tartrate 41.83 (3.01) $\mu g.g^{-1}$ (dry wt.); antimony trioxide 29.45 (2.11) $\mu g.g^{-1}$; potassium hexahydroxyantimonate 6.13 (2.87) $\mu g.g^{-1}$. Figure in parentheses represent standard deviation of three replicate incubations.

4.3.8 GC-AAS analysis of involatile antimony species present in culture supernatant

GC-AAS analysis of derivatised culture supernatant from *C.humicolus* incubations that were supplied with potassium antimony tartrate as biotransformation substrate revealed the presence of monomethylstibine, dimethylstibine, and trimethylstibine. The precise speciation of the pre-derivatised involatile methylantimony species in the liquid phase cannot be elucidated with the hydride generation-GC-AAS technique; only the degree of alkylation of the metal can be ascertained. It is reasonable to conjecture however, that within an aerobic growth situation these species exist in an oxidised form, i.e. as monomethylstibonic acid, dimethylstibinic acid and trimethylantimony oxide, such as is described for biomethylation of arsenic by *C.humicolus*.[5] The identification of trimethylantimony oxide as the trimethylated species was confirmed by HPLC-hydride generation-AFS analysis.

In contrast to *S.brevicaulis*,[9] only low levels of involatile methylantimony species were produced during the linear growth phase of *C.humicolus* (Table 4.8 and Figure 4.9). The amount of methylated antimony species detected in culture supernatant increased during the stationary phase at a rate of 0.6 $ng.ml^{-1}$ per day. The rate of formation continued to increase, at a rate of 5.7 $ng.ml^{-1}$ per day, with a concurrent decrease in culture absorbance at 600 nm from 4.2 to 1.6 units. No associated change in gross morphology was noted, e.g. from mycelial to fragmented hyphae or yeast form. It is possible that the increase in methylantimony formation rate in later stages of the incubation is due to release of involatile methylantimony species during cell lysis, or may be a result of an increase in biomethylation capability during the death phase. Indeed, when potassium antimony tartrate was added to the incubation during the stationary phase of growth (day 7) (Figure 4.9) the rate of production of involatile methylated antimony species (day 11 - 21) was not significantly different (at 95% confidence level) to that observed when the metal was present from the start of the incubation. 5.7 $ng.ml^{-1}$ per day and 5.5 $ng.ml^{-1}$ per day respectively. The increase in antimony biomethylation during the death phase may suggest that the cells have an excess of NADH (i.e. the reductive charge $NADH/(NAD^{+}$ NADH) which is normally held at 0.05 starts to increase. Since methylation is a reductive process and the cells are not actively biosynthesising macromolcules, the cells may be methylating antimony or arsenic as a means of dissipating NADH and maintaining catabolic reduction charge at 0.05.

Table 4.8 Formation of involatile methylantimony species during incubation of *C.humicolus* with potassium antimony tartrate (50mg.l^{-1}) and the protein synthesis inhibitor cycloheximide.

Treatment	Rate of formation (ng.ml^{-1} per day)						Total amount of methylantimony species after 21 days incubation (ng.ml^{-1})
	Day 0 → 11			Day 11→ 21			
	MeSb	Me_2Sb	Me_3Sb	MeSb	Me_2Sb	Me_3Sb	
Sb @ day 0	0.0	0.2	0.4	0.3	1.9	3.5	68.9 (5.1)
Sb @ day 7	0.0	0.0	0.0	0.3	1.3	3.9	60.8 (5.5)
CH @ day 3	0.0	0.0	0.2	0.0	0.0	0.2	1.9 (1.5)
CH @ day 7	0.0	0.0	0.0	0.2	1.5	2.7	49.2 (5.3)

Additions of potassium antimony tartrate (Sb) and cycloheximide (CH) were made at times shown. Antimony was present from t = 0 in incubations supplied with cycloheximide; figure in parentheses are standard deviations based on three replicate culture incubations; there was no statistical difference between the means at the 95% confidence level when antimony was added at day 0 or day 7, the mean total amounts of methylantimony in incubations with and without cycloheximide were statistically different at the 95% confidence level.

4.3.9 Addition of cycloheximide – effect on formation of involatile methylantimony species

The addition of cycloheximide at t = 3 days to culture incubations containing potassium antimony tartrate from t = 0, resulted in the almost complete absence of involatile methylantimony species in culture supernatant (Table 4.8 and Figure 4.10). Addition of cycloheximide to culture incubations at t = 7 days resulted in a decrease in the rate of production of involatile antimony species. The total amount of methylantimony observed after 21 days incubation was 49.2 ng.ml^{-1} compared to 68.9 ng.ml^{-1} in the absence of cycloheximide. These data demonstrate the inhibitory effect of cycloheximide and indicate that the rate of production of methylantimony is dependant upon protein synthesis. The data further demonstrates that the capability of *C.humicolus* to biomethylate antimony is not a feature of the linear growth phase, but is developed, at least in part by the mid-stationary phase.

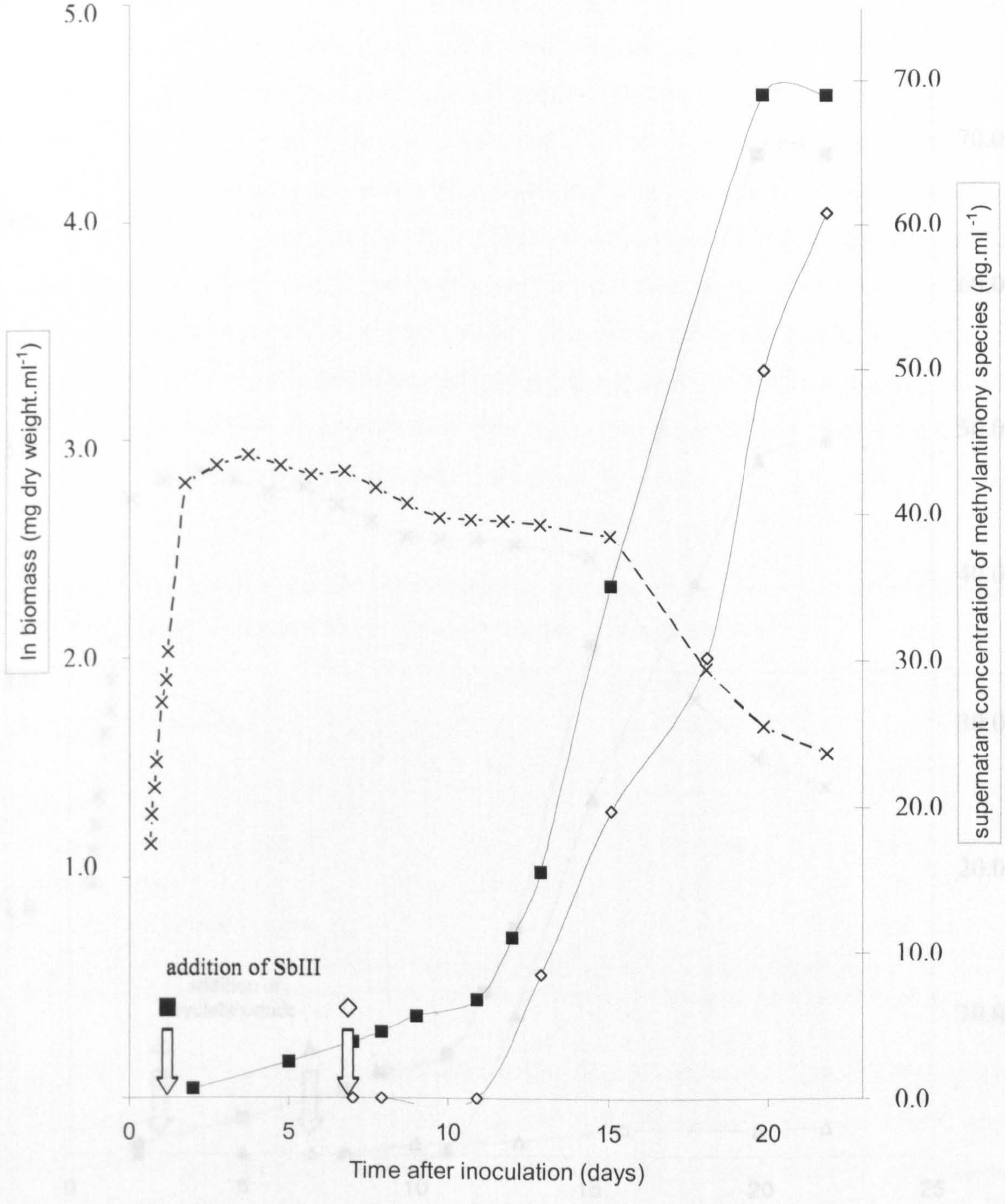


Figure 4.9 Production profile of total involatile methylantimony species; (■) Potassium antimony tartrate present from t = 0, (◇) addition of potassium antimony tartrate at day 7, (x) = biomass profile.

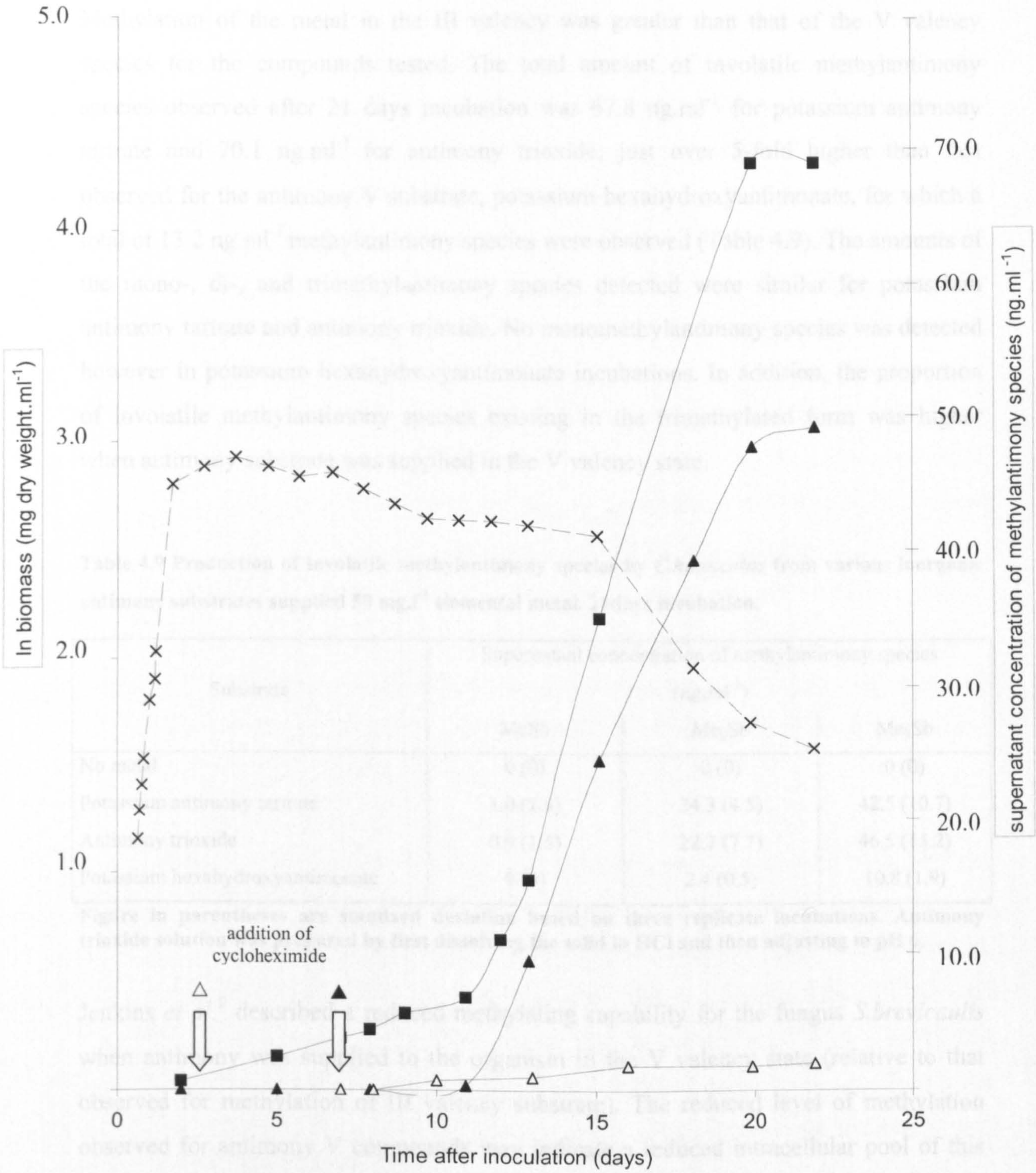


Figure 4.10 Effect of cycloheximide on methylation of antimony by *C.humicolus*; (■) no cycloheximide, (△) cycloheximide addition at t= 3 days, (▲) cycloheximide addition at t= 7 days, Potassium antimony tartrate was present in all incubations from t = 0. (x) biomass profile.

4.3.10 Methylation of various substrates

C.humicolus was shown to methylate a number of inorganic antimony substrates. Methylation of the metal in the III valency was greater than that of the V valency species for the compounds tested. The total amount of involatile methylantimony species observed after 21 days incubation was 67.8 $ng.ml^{-1}$ for potassium antimony tartrate and 70.1 $ng.ml^{-1}$ for antimony trioxide; just over 5-fold higher than that observed for the antimony V substrate, potassium hexahydroxyantimonate, for which a total of 13.2 $ng.ml^{-1}$ methylantimony species were observed (Table 4.9). The amounts of the mono-, di-, and trimethylantimony species detected were similar for potassium antimony tartrate and antimony trioxide. No monomethylantimony species was detected however in potassium hexahydroxyantimonate incubations. In addition, the proportion of involatile methylantimony species existing in the trimethylated form was higher when antimony substrate was supplied in the V valency state.

Table 4.9 Production of involatile methylantimony species by *C.humicolus* from various inorganic antimony substrates supplied 50 $mg.l^{-1}$ elemental metal. 21days incubation.

Substrate	Supernatant concentration of methylantimony species ($ng.ml^{-1}$)		
	MeSb	Me_2Sb	Me_3Sb
No metal	0 (0)	0 (0)	0 (0)
Potassium antimony tartrate	1.0 (1.8)	24.3 (4.5)	42.5 (10.7)
Antimony trioxide	0.9 (1.5)	22.7 (7.7)	46.5 (15.2)
Potassium hexahydroxyantimonate	0 (0)	2.4 (0.5)	10.8 (1.9)

Figure in parentheses are standard deviation based on three replicate incubations. Antimony trioxide solution was prepared by first dissolving the solid in HCl and then adjusting to pH 6.

Jenkins *et al.*[9] described a reduced methylating capability for the fungus *S.brevicaulis* when antimony was supplied to the organism in the V valency state (relative to that observed for methylation of III valency substrate). The reduced level of methylation observed for antimony V compounds may indicate a reduced intracellular pool of this form, for example as a result of lower cellular uptake rates. The Challenger mechanism for biomethylation of arsenic, which is generally accepted to be applicable to antimony, describes an initial reduction of antimony V valency to the III which may also contribute to the reduced methylation seen with antimony V substrates.

4.3.11 Effect of cell lysis upon supernatant levels of involatile methylantimony species

Lysis of early stationary phase-late linear phase cells by lyticase and sonication resulted in a marked increase in the amount of methylated antimony species detected in the culture supernatant by hydride generation-GC-AAS (Table 4.10). This indicates that release of methylantimony species from biomass may account for the apparent biphasic production. A total of 631.4 $ng.ml^{-1}$ methylantimony was detected in culture supernatant after lysis compared to 3.7 $ng.ml^{-1}$ in pre-lysis supernatant. Amounts of involatile methylantimony species detected were higher even than supernatant levels normally seen after 19 days incubation (69.3 $ng.ml^{-1}$), suggesting a cellular accumulation of the methylated species which would seem to belie the supposition that methylation of inorganic antimony to more volatile or mobile species is a resistance mechanism. Although sonicated cell suspensions were subsequently centrifuged to remove biomass, it is entirely possible that fragments of cell wall/membrane will have been retained within the supernatant. It is difficult therefore to distinguish between an intracellular, and wall/membrane bound location for the methylantimony species. Surface binding/uptake studies (section 4.3.7) of trimethylantimony oxide however, revealed no surface binding or other association between this compound and *C.humicolus* biomass. This suggests that the enhanced supernatant levels of methylantimony species noted upon cell lysis, is intracellular in origin.

The ratio of methylantimony species, mono-; di-; tri-, was markedly different pre- and post-lysis treatment. Up to 89% of the total involatile methylantimony species detected pre-lysis was trimethylantimony and no monomethylantimony species were detected. The proportion of trimethylantimony species fell to 52.5% after lysis, with dimethylantimony and monomethylantimony species comprising the remaining 39.4 and 8% respectively. This would seem to suggest that the trimethylantimony form is the major exported species, which may indicate that monomethylantimony and dimethylantimony species are precursors in the intracellular processing of inorganic antimony to the trimethylated form, as is described for the Challenger mechanism of arsenic biomethylation. Interestingly, the ratio of methylated antimony species is very similar between the lysed treatment and the 19-day incubation. The increased proportion of monomethylantimony and dimethylantimony species noted in the 19-day incubation is possibly due to natural cell lysis as the culture ages, releasing partially methylated

species to the supernatant. It is not possible to comment upon cellular export of methylated antimony since studies with transport inhibitors were not performed. However, the elevated proportion of trimethylantimony species in culture supernatant compared to intracellular levels would appear to indicate a preferential movement of the trimethylated species over monomethylantimony and dimethylantimony, suggesting that specific transporters are involved.

Table 4.10 Supernatant concentration of methylantimony species is enhanced upon cell lysis.

Sample	Supernatant concentration of methylantimony species ($ng.ml^{-1}$)		
	MeSb	Me_2Sb	Me_3Sb
Pre-lysis treatment, 6 day incubation	nd	0.4 (0.2)	3.3 (1.1)
Post-lysis treatment, 6 day incubation	50.9 (4.5)	249.0 (15.7)	331.5 (15.6)
No lysis (control reference), 19 day incubation	2.1 (1.2)	28.8 (2.7)	38.4 (3.2)

nd = not detected (< 20 $pg.ml^{-1}$); figure in parentheses are standard deviations based on three replicate culture incubations. Lysis was achieved through physico-enzymatic means. i.e. incubation of cell culture in presence of lyticase with subsequent sonication.

4.3.12 Effect of initial antimony concentration on methylation of antimony by *C.humicolus* and *S.brevicaulis*

The total amount of methylantimony species detected in culture incubations of *C.humicolus* was positively correlated with initial substrate level up to a maximum substrate loading of 300 $mg.l^{-1}$ for the compound potassium antimony tartrate. Further increases in substrate levels resulted in a decrease in the amount of methylation observed (from 183.5 $ng.ml^{-1}$ at 300 $mg.l^{-1}$ to 108.6 $ng.ml^{-1}$ at 1000 $mg.l^{-1}$), possibly as a result of cellular toxicity (Figure 4.11). The ratio of methylantimony species was observed to be dependent upon initial substrate concentration. Trimethylantimony was the predominant species present in culture supernatant at relatively low inorganic antimony concentrations (< 50 $mg.l^{-1}$). However, as substrate levels increased, the proportion of methylated antimony existing in the dimethylantimony form increased. The proportion of dimethylantimony species subsequently decreased when total methylantimony amounts fell in response to elevated inorganic substrate levels. The maximum proportion of dimethylantimony species observed in the incubation was 51% when substrate levels were 300 $mg.l^{-1}$ potassium antimony tartrate. These data may be explained by considering that the transformation of dimethylantimony to the trimethylated form is the rate limiting step; when flux of antimony into the pathway is

sufficiently high, enzymatic transformation of di- to trimethylantimony is no longer able to keep pace resulting in an accumulation of the dimethylated species. The enzymes involved in the biomethylation of arsenic are the most likely catalysts for antimony methylation. Cullen *et al.* [32] noted a similar phenomenon for the biomethylation of arsenic by *C.humicolus*. They reported that biotransformation of dimethylarsinic acid to trimethylarsenic compounds was a slow process compared to methylation of arsenic III, arsenic V or monomethylarsonic acid.

Transformation of inorganic substrate to involatile methylantimony species by *S.brevicaulis* displayed a similar profile to *C.humicolus* (Figure 4.11). As with *C.humicolus*, maximum methylantimony formation (319.1 $ng.ml^{-1}$) was detected at 300 $mg.l^{-1}$ inorganic antimony substrate loading. This is approximately two-fold higher compared to *C.humicolus*. In contrast to *C.humicolus* incubations, dimethylated antimony species were never present as the predominant methylantimony form in *S.brevicaulis* incubations. However, the ratio of di- to trimethylated species was highest at maximum supernatant levels of methylantimony. The rate of antimony volatilisation by *S.brevicaulis* has previously been reported to be positively correlated with the concentration of inorganic substrate present in the incubation.[9] No speciation was however reported, since the trapping procedure involved destruction of volatile antimony species in nitric acid.

4.3.13 Effect of arsenic on methylation of antimony by *C.humicolus*

Pre-incubation of *C.humicolus* to mid-stationary phase with arsenic III as sodium arsenite resulted in a significant (at 95% confidence level) increase in the rate of formation of involatile methylantimony species. In addition, the trimethylated form was the sole methylantimony species detected. A maximum rate of formation of 11.5 $ng.ml^{-1}$ per day was observed when biomass was pre-incubated with 50 $mg.l^{-1}$ arsenic III (Table 4.10 and Figure 4.12). Pre-incubation with 100 $mg.l^{-1}$ arsenic III did not increase the rate of biomethylation to as great an extent as 50 $mg.l^{-1}$ arsenic III. This may be due to toxicity of the metal at this concentration; resistance studies (section 4.3.2) demonstrated that inclusion of 100 $mg.l^{-1}$ arsenic III induced an inhibitory effect on growth of *C.humicolus*. Pre-incubation with antimony III as potassium antimony tartrate did not stimulate a higher rate of biomethylation. These data indicate that whilst

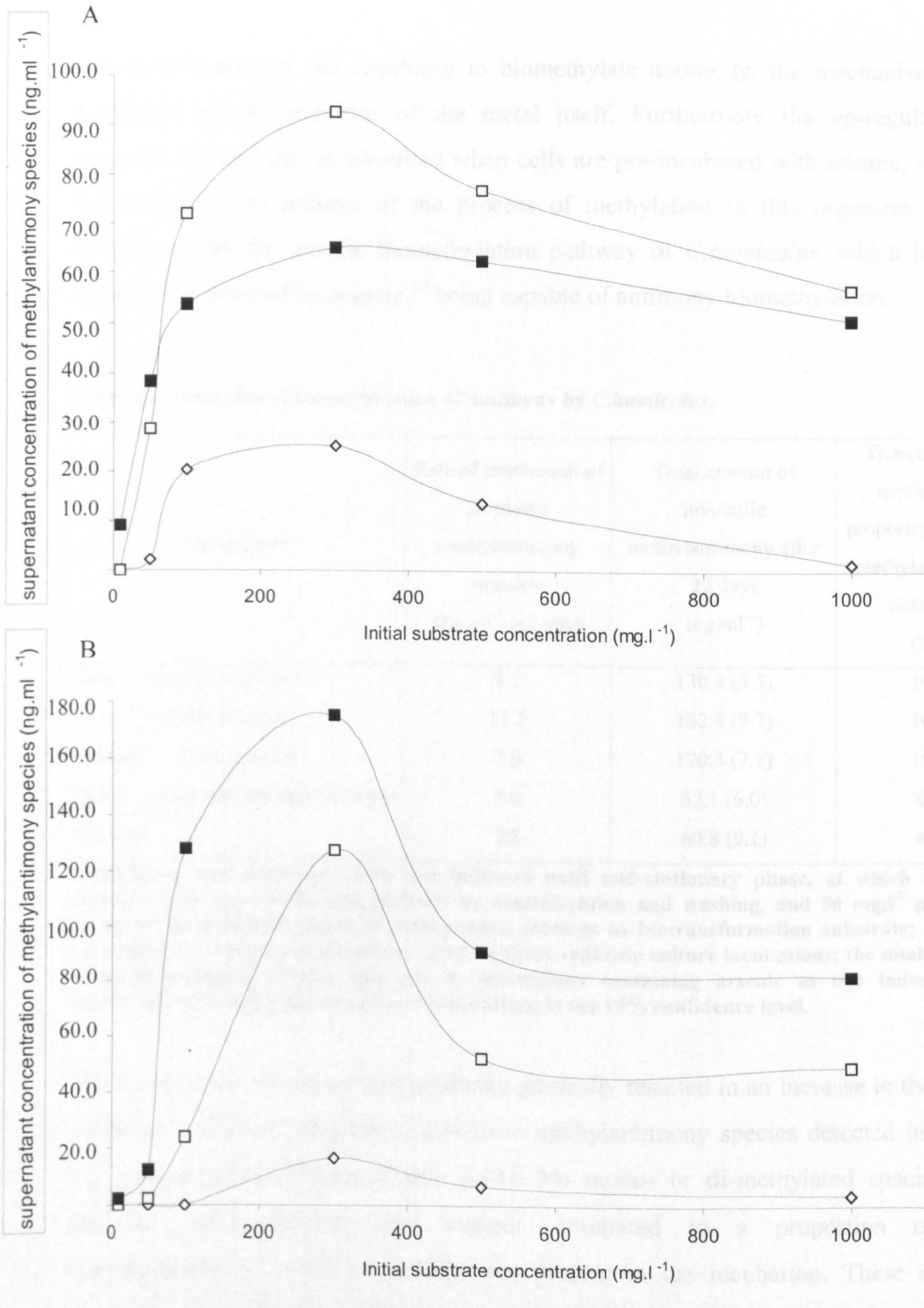


Figure 4.11 Effect of initial substrate concentration (supplied as potassium antimony tartrate) on final supernatant concentrations of methylated antimony species in incubations (19 days) of (A) *C.humicolus*, and (B) *S.brevicaulis*. (■) trimethyl; (□) dimethyl; (◇) monomethyl.

C.humicolus possess the capability to biomethylate antimony, the mechanism is not stimulated by the presence of the metal itself. Furthermore the up-regulation of antimony biomethylation observed when cells are pre-incubated with arsenic, indicates that arsenic is an inducer of the process of methylation in this organism. This is consistent with the arsenic biomethylation pathway of *C.humicolus*, which has been shown to be induced by arsenic,[40] being capable of antimony biomethylation.

Table 4.11 Induction of biomethylation of antimony by *C.humicolus*.

Test inducer	Rate of production of involatile methylantimony species ($ng.ml^{-1}$ per day)	Total amount of involatile methylantimony after 22 days ($ng.ml^{-1}$)	Trimethylated species as a proportion of total methylantimony detected (%)
$10mg.l^{-1}$ sodium arsenite	8.2	130.4 (5.5)	100
$50mg.l^{-1}$ sodium arsenite	11.5	182.4 (9.7)	100
$100mg.l^{-1}$ sodium arsenite	7.6	120.3 (7.1)	100
$50mg.l^{-1}$ potassium antimony tartrate	3.6	52.1 (6.0)	66
No metal	3.8	60.8 (9.1)	64

C.humicolus was incubated with test inducers until mid-stationary phase, at which time test inducers were separated from biomass by centrifugation and washing, and 50 $mg.l^{-1}$ potassium antimony tartrate was added to resuspended biomass as biotransformation substrate; figure in parentheses are standard deviations based on three replicate culture incubations; the total amounts of methylantimony species detected in incubations containing arsenic as test inducer were statistically different from no inducer incubations at the 95% confidence level.

The co-presence of arsenic and antimony generally resulted in an increase in the rate of production and total amount of involatile methylantimony species detected in culture supernatants after 13-days (Table 4.11). No mono- or di-methylated species were observed when arsenic was present, compared to a proportion of 42% dimethylantimony when no arsenic was present in the incubation. These data are consistent with induction of antimony biomethylation by arsenic.

The up-regulation of antimony biomethylation when arsenic was co-present with potassium antimony tartrate was more marked when arsenic was supplied in the III valency state than the V. Both these compounds are known to be methylated by *C.humicolus* [13] with metabolism of arsenic V requiring a prior reduction step. The observed effect of less up-regulation from arsenic V than arsenic III may therefore be a

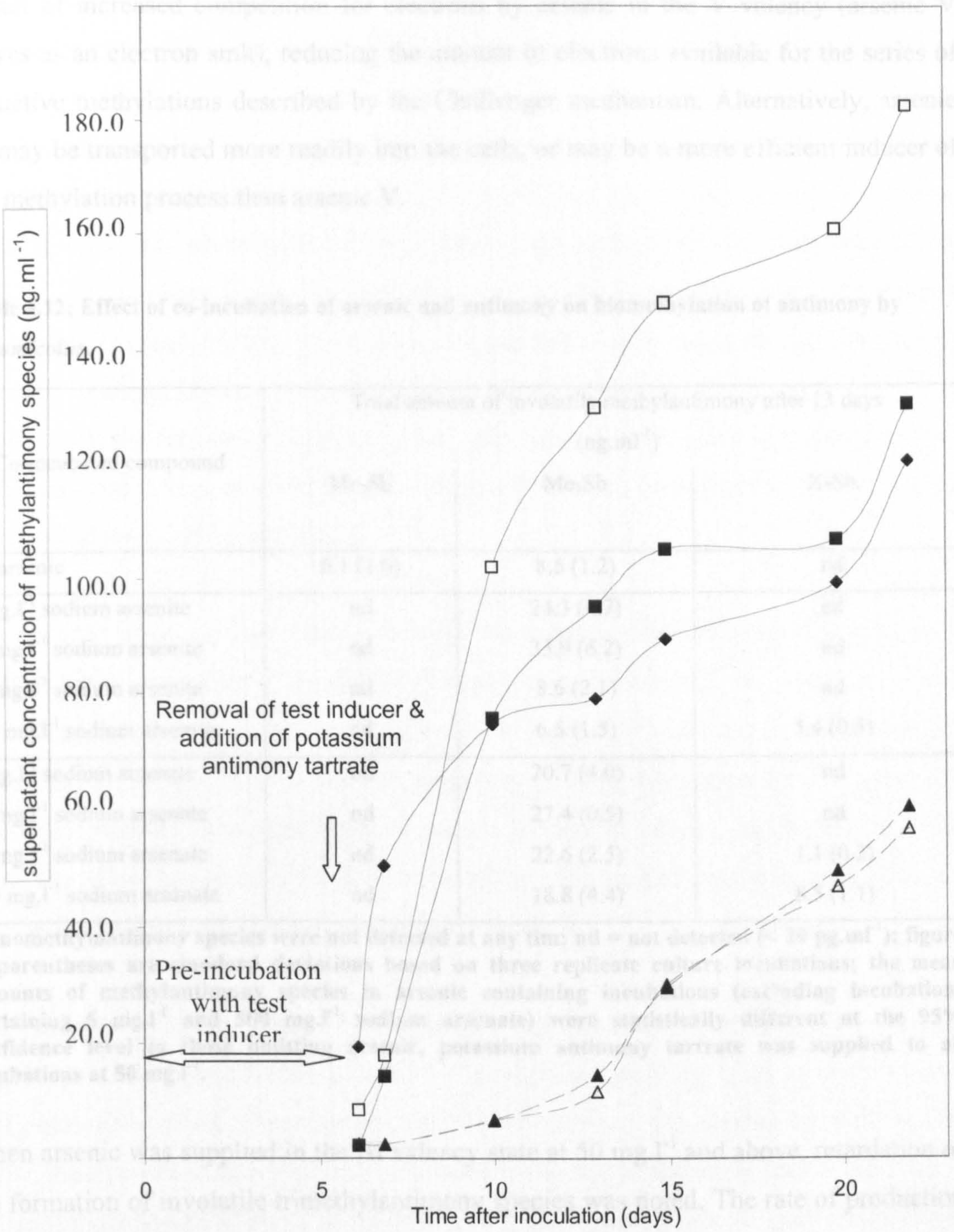


Figure 4.12 Effect of preconditioning *C.humicolus* with sodium arsenite or potassium antimony tartrate on formation of involatile methylantimony species. Cells were incubated with (■) 10 mg.l^{-1} arsenic III, (□) 50 mg.l^{-1} arsenic III, (◆) 100 mg.l^{-1} arsenic III, (▲) 50 mg.l^{-1} antimony III, (△) no metal addition, till mid stationary phase at which point residual inducer metal was removed by centrifugation and washing of cells, and 50 mg.l^{-1} potassium antimony tartrate biotransformation substrate was added to re-suspended biomass.

result of increased competition for electrons by arsenic in the V valency (arsenic V serves as an electron sink), reducing the amount of electrons available for the series of reductive methylations described by the Challenger mechanism. Alternatively, arsenic III may be transported more readily into the cells, or may be a more efficient inducer of the methylation process than arsenic V.

Table 4.12; Effect of co-incubation of arsenic and antimony on biomethylation of antimony by *C.humicolus*.

Co-incubated compound	Total amount of involatile methylantimony after 13 days ($ng.ml^{-1}$)		
	Me_2Sb	Me_3Sb	X-Sb
No arsenic	6.1 (1.0)	8.5 (1.2)	nd
5 $mg.l^{-1}$ sodium arsenite	nd	24.3 (4.7)	nd
10 $mg.l^{-1}$ sodium arsenite	nd	33.9 (6.2)	nd
50 $mg.l^{-1}$ sodium arsenite	nd	8.6 (2.1)	nd
500 $mg.l^{-1}$ sodium arsenite	nd	6.5 (1.5)	5.4 (0.5)
5 $mg.l^{-1}$ sodium arsenate	nd	20.7 (4.0)	nd
10 $mg.l^{-1}$ sodium arsenate	nd	27.4 (0.5)	nd
50 $mg.l^{-1}$ sodium arsenate	nd	22.6 (2.5)	1.1 (0.2)
500 $mg.l^{-1}$ sodium arsenate	nd	18.8 (4.4)	8.5 (1.1)

Monomethylantimony species were not detected at any tim; nd = not detected (< 20 $pg.ml^{-1}$); figure in parentheses are standard deviations based on three replicate culture incubations; the mean amounts of methylantimony species in arsenic containing incubations (excluding incubations containing 5 $mg.l^{-1}$ and 500 $mg.l^{-1}$ sodium arsenate) were statistically different at the 95% confidence level to those omitting arsenic, potassium antimony tartrate was supplied to all incubations at 50 $mg.l^{-1}$.

When arsenic was supplied in the III valency state at 50 $mg.l^{-1}$ and above, retardation of the formation of involatile trimethylantimony species was noted. The rate of production fell to 0.5 $ng.ml^{-1}$ per day, 22% less than when no arsenic was present in the incubation. A similar reduction in antimony biomethylation above 10 $mg.l^{-1}$ arsenic was observed when arsenic was supplied in the V valency form. The reduction in antimony biomethylation was not as marked as that seen in arsenic III incubations and did not fall below the "no arsenic incubation" level. When arsenic III was supplied to incubations at 500 $mg.l^{-1}$ a peak with retention time 2.3 minutes (bp = 89.5°C) was observed, that is, after the elution of trimethylated antimony species (Figure 4.13). The amount of the unknown antimony compound detected does not account for the total reduction in

trimethylated antimony species that was observed when arsenic levels were increased, and may not be a further metabolised product of the antimony biomethylation pathway.

The deleterious effect of arsenic upon trimethylantimony species formation at higher concentrations could be due to a number of reasons; (i) cellular toxicity of arsenic (section 4.3.2), for which arsenic III has been shown to be more toxic than arsenic V; a reduction in biomass levels, or occupation of cellular metabolism with homeostasis maintenance at such high levels of arsenic would likely result in an overall reduction of antimony biomethylation; (ii) competitive inhibition of binding sites by arsenic III; it is possible that the reduction in antimony biomethylation observed as co-incubated arsenic levels increase, arises as a result of competitive inhibition of methyltransferase binding sites for antimony III by arsenic III.

The reduced effect of high arsenic V levels on antimony biomethylation compared to arsenic III is consistent with the III valency metalloid being the substrate of the methyltransferase enzyme and prior reduction of arsenic V to arsenic III being required before inhibition can take place. The reduction in methylantimony species formation that was observed for higher levels of arsenic V may, in addition to the electron sink effect described before, be a result of competitive inhibition of the methyltransferase by arsenic III produced by cellular reduction of arsenic V.

The unknown antimony peak (t = 2.3 minutes) seen when arsenic III was present at 500 $mg.l^{-1}$ was also observed when arsenic V was supplied at 50 $mg.l^{-1}$ to incubations, and was noted to increase when the arsenic V concentration was further elevated to 500 $mg.l^{-1}$. Analysis of samples using an arsenic specific lamp resulted in loss of the peak at 2.3 min; dimethylarsine and trimethylarsine were however observed. This demonstrates that peak at 2.3 min. is not a product of hydride generation of an arseno-antimony compound. Attempts to elucidate the identity of X-Sb by GC-MS were unsuccessful. Despite sample preparation in exactly the same way as for GC-AAS analysis, no antimony-containing peak could be detected after the elution of trimethylstibine. It is a possibility that the unknown compound interacted with the column packing (CP-sil 8CB) and was not eluted, or was more sensitive to thermo-oxidation in the injector port (140^{o}C) than the simple methylantimony compounds.

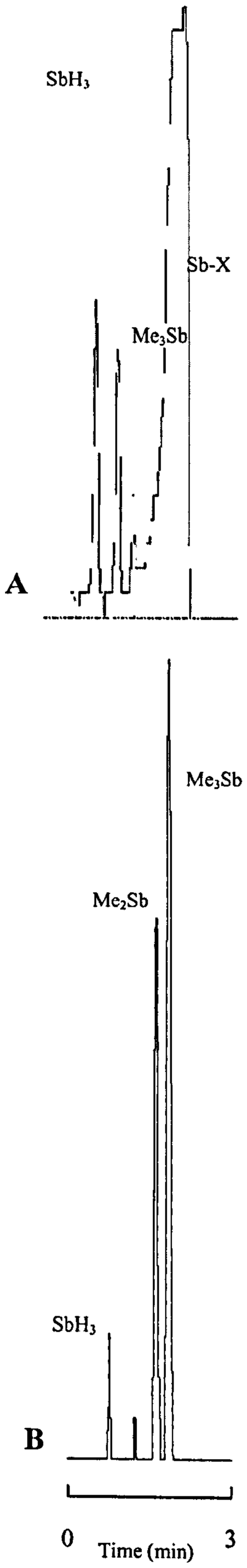


Figure 4.13 Typical GC-AAS chromatograms of (A) hydride generated culture supernatant from *C.humicolus* incubation amended with 50 mg.l^{-1} potassium antimony tartrate and 50 mg.l^{-1} sodium arsenate showing presence of additional antimony containing peak eluted after trimethylantimony, and (B) volatile antimony standards produced by hydride generation of trimethylantimony dichloride.

4.3.14 Hydride generation-GC-AAS analysis of involatile arsenic species

GC-AAS analysis of hydride generated culture supernatant from *C.humicolus* incubations supplied with 1 $mg.l^{-1}$ arsenic III as sodium arsenite confirmed the arsenic biomethylating capability of this organism as first described by Cox and Alexander.[13] Involatile di- and trimethylarsenic species were identified in all samples, with the trimethylated form predominating (Table 4.12). Cullen *et al.* [32] also describe the detection of involatile dimethylated arsenic species in culture supernatants of *C.humicolus*, however no quantification of species was presented.

Up to 8.0% of the total inorganic arsenic substrate supplied to incubations was methylated by C.humicolus. This is far higher than the degree of methylation observed from antimony substrates. At 1 $mg.l^{-1}$ antimony III no methylantimony species were observed and at 10 $mg.l^{-1}$ antimony III less than 0.1% of the inorganic substrate was methylated. These data, coupled with the reports of antimony and arsenic biomethylation by the fungus S.brevicaulis,[9] reinforce the view that biomethylation of antimony is less favourable than that of its element arsenic.

Table 4.13 Comparison of formation of involatile methylarsenic and methylantimony species from inorganic metal substrate by *C.humicolus*. Hydride generation-GC-AAS analysis.

Substrate	Total amount of involatile methyl species after 19 days ($ng.ml^{-1}$)		Proportion of substrate methylated (%)	
	Me_2M	Me_3M	Me_2M	Me_3M
Sodium arsenite ($1mg.l^{-1}$)	2.1 (0.9)	77.7 (2.4)	0.21	7.80
Potassium antimony tartrate ($1mg.l^{-1}$)	nd	nd	-	-
Potassium antimony tartrate ($10mg.l^{-1}$)	nd	9.1 (0.5)	-	0.09

nd = not detected (< 20 $pg.ml^{-1}$); figure in parentheses are standard deviations based on three replicate culture incubations; no monomethylated antimony or arsenic species were not detected at any time; M = metal being As for incubations supplied with inorganic arsenic substrate and Sb for those supplied with antimony

4.3.15 HPLC-hydride generation-AFS analysis of involatile antimony species

Analysis of culture supernatants by HPLC-separation with subsequent derivatisation of hydride forming species with sodium borohydride and detection by atomic fluorescence spectrometry (AFS) (antimony specific) revealed the presence of three antimony containing peaks. Retention times were typically 0.9, 2.2 and 2.4 minutes (Figure 4.14).

Analysis of trimethylantimony oxide standard by HPLC-hydride generation-AFS resulted in the appearance of two peaks with retention times 0.9 and 2.4 minutes, which were tentatively assigned as trimethylantimony dihydroxide (0.9 min.) and dimerised trimethylantimony dihydroxide (2.4 min.). In the absence of further methylantimony standards, it is impossible to definitively identify the sample peak observed at 2.2 minutes. From a comparison of HPLC chromatograms with spectra obtained by GC-AAS analysis of identical culture incubations, it is reasonable to suppose that this is a dimethylated antimony species, possibly in dimerised form. The absence of a discrete peak attributable to the monomeric dimethylantimony species earlier in the chromatogram is most probably due to lack of resolution since the peak at 0.9 minutes (trimethylantimony dihydroxide) occurs within or shortly after the solvent front. As described before (section 2.1) inorganic antimony (100 $\mu g.ml^{-1}$) could be efficiently removed (final concentration <20 $pg.ml^{-1}$) from cultures supernatants by passing through a basic alumina column buffered with $0.1 mol.l^{-1}$ potassium acetate (pH 7.5). Since (i) incubations for HPLC analysis were never supplied with more than 50 $\mu g.ml^{-1}$. inorganic substrate and (ii) inorganic antimony III species could not be eluted from the HPLC column using this methodology, it may be concluded that the peaks observed comprise organoantimony species.

Assuming that the peaks at 2.2 and 2.4 minutes are methylated antimony species, then comparison of amounts detected compare favourably with those observed by GC-AAS analysis of identical incubations (Table 4.13). For example, a total of 67.3 $mg.ml^{-1}$ methylantimony species was detected by HPLC-hydride generation-AFS analysis of *C.humicolus* incubations supplied with 50 $mg.l^{-1}$ potassium antimony tartrate, compared to 69.3 $ng.ml^{-1}$ by hydride generation-AAS analysis of identical incubations.

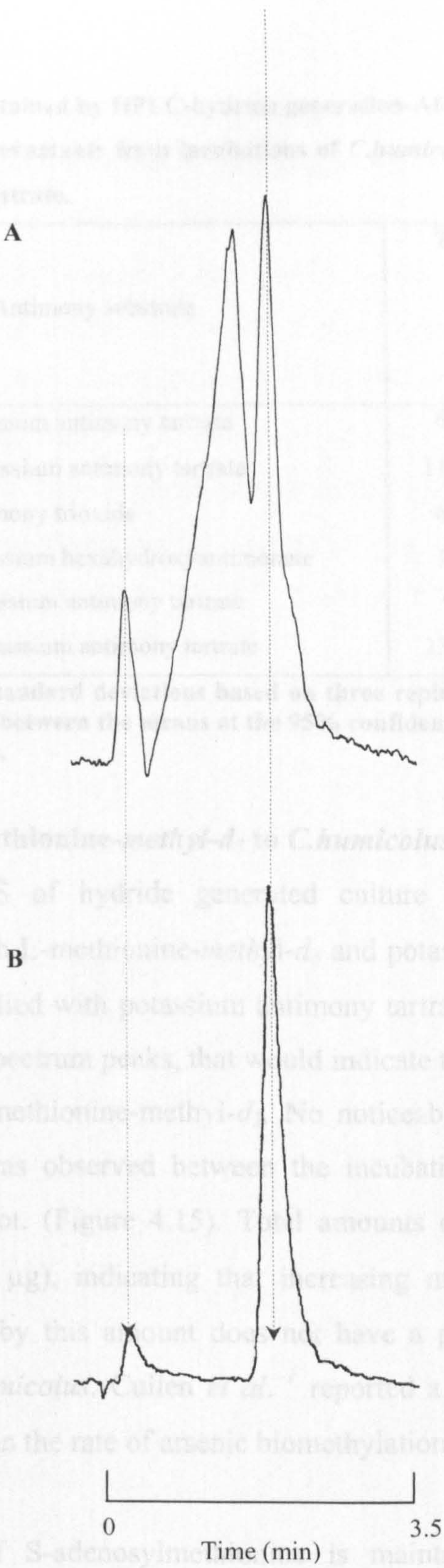


Figure 4.14 Typical chromatograms obtained from HPLC-hydride generation-AFS analysis of culture supernatant from *C.humicolus* incubations supplied with potassium antimony tartrate. (A) *C.humicolus* + 50 mg.l^{-1} potassium antimony tartrate; retention times of peaks: 0.9, 2.2 and 2.4 minutes. (B) Chromatogram of trimethylantimony oxide (1 ng) standard run under identical chromatographic conditions, peak retention times: 0.9 and 2.4 minutes (possibly due to degradation).

Table 4.14 Typical values obtained by HPLC-hydride generation-AFS and hydride generation-GC-AAS analysis of culture supernatants from incubations of *C.humicolus* and *S.brevicaulis* supplied with inorganic antimony substrate.

Organism / Antimony substrate	Total methylantimony species detected ($ng.ml^{-1}$)	
	HPLC	GC-AAS
C.humicolus + 50 $mg.l^{-1}$ potassium antimony tartrate	67.3 (2.1)	69.3 (3.8)
C.humicolus + 100 $mg.l^{-1}$ potassium antimony tartrate	118.8 (16.8)	146.0 (6.9)
C.humicolus + 50 $mg.l^{-1}$ antimony trioxide	67.6 (3.6)	70.1 (15.1)
C.humicolus + 50 $mg.l^{-1}$ potassium hexahydroxyantimonate	10.0 (2.6)	13.2 (1.9)
S.brevicaulis + 50 $mg.l^{-1}$ potassium antimony tartrate	9.0 (0.8)	11.4 (0.4)
S.brevicaulis + 100 $mg.l^{-1}$ potassium antimony tartrate	131.2 (24.5)	151.7(6.9)

Figure in parentheses are standard deviations based on three replicate culture incubations; there was no statistical difference between the means at the 95% confidence level (excluding *S.brevicaulis* + 50 $mg.l^{-1}$ PAT incubation).

4.3.16 Addition of L-methionine-*methyl-d_3* to *C.humicolus* incubations

Comparison by GC-MS of hydride generated culture supernatants from culture incubations supplied with L-methionine-*methyl*-d_3 and potassium antimony tartrate and culture incubations supplied with potassium antimony tartrate alone, did not reveal the presence of extra mass spectrum peaks, that would indicate the incorporation of labelled methyl groups from L-methionine-methyl-d_3. No noticeable difference in the overall fragmentation pattern was observed between the incubations supplied with labelled methionine and those not. (Figure 4.15). Total amounts of trimethylstibine detected were comparable (0.49 μg), indicating that increasing media concentrations of the amino acid methionine by this amount does not have a positive effect on antimony biomethylation by *C.humicolus*. Cullen *et al.* [1] reported a similar lack of stimulatory effect by methionine upon the rate of arsenic biomethylation by this organism.

Intracellular balance of S-adenosylmethionine is maintained by product induced negative feedback and induction of degradation. That is to say, that S-adenosylmethionine represses its own synthesis *via* methionine adenosylmethyltransferase and induces its metabolism to homocysteine and methionine by S-adenosylmethionine;homocysteine methyltransferase.[41] Elevation of cellular methionine levels will have the initial effect of increasing the concentration of S-adenosylmethionine, but this effect will be short lived due to inhibition of further biosynthesis by inhibition of methionine adenosylmethyltransferase and induction of

adenosylmethionine;homocysteine methyltransferase, which degrades S-adenosylmethionine *via* homocysteine to methionine. Excess methionine can enter the tricarboxylic acid cycle *via* succinyl CoA. The report of an increase in antimony biomethylation for the fungus *S.brevicaulis* when media levels of methionine were increased [27] may suggest that methionine is a rate limiting nutrient for *S.brevicaulis* and that the increase in antimony biomethylation observed arises therefore as a result of increased biomass concentration. A four-fold increase in the total amount of trimethylantimony species was observed compared to incubations omitting the amino acid. The levels of trimethylantimony compounds in incubations containing L-methionine were approximately 20 ng Sb ml^{-1} compared to approximately 5 ng Sb ml^{-1} in incubations not containing L-methionine.[27]

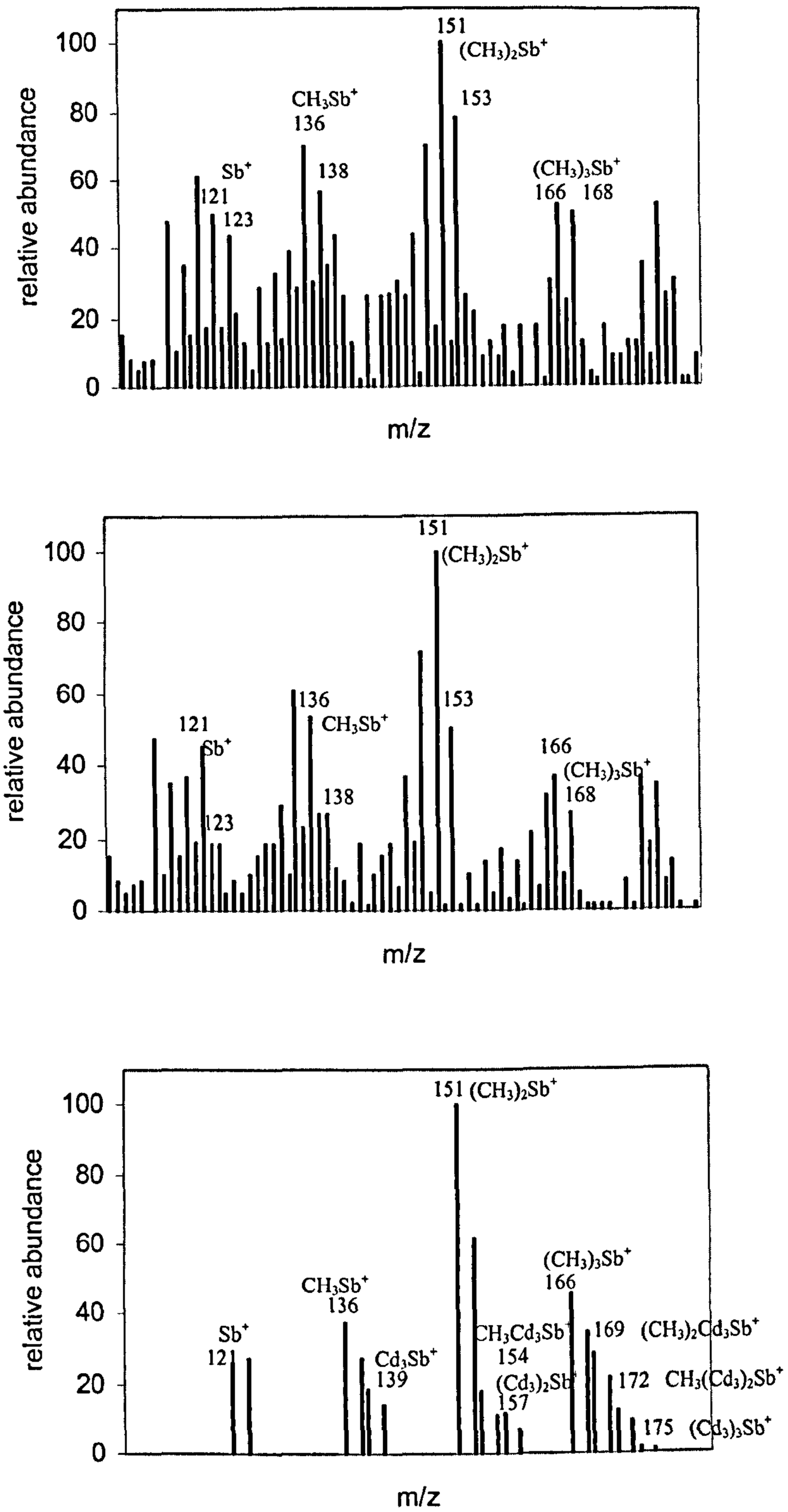


Figure 4.15 Mass spectrum of hydride generated trimethylantimony from *C.humicolus* culture incubations. (A) 50 mg.l^{-1} potassium antimony tartrate. (B) 50 mg.l^{-1} potassium antimony tartrate + 1.3 mmole.l^{-1} L-methionine-*methyl-d$_3$* showing no incorporation of *methyl-d$_3$*. (C) Theoretical mass spectrum of labelled trimethylantimony based on (i) NIST spectrum for trimethylantimony and (ii) reported % incorporation of *methyl-d$_3$* to trimethylarsenic oxide during biomethylation of inorganic arsenic by *C.humicolus* [32].

4.4 Discussion

The anamorphic basidomycetous yeast *Cryptococcus humicolus* was shown to possess the capability to biomethylate inorganic antimony compounds. This microorganism is a known biomethylator of arsenic.[13, 14] In addition to the detection of involatile monomethylantimony, dimethylantimony and trimethylantimony species in culture supernatants, volatile trimethylstibine and stibine (SbH_3) of biogenic origin were detected in culture headspace gases. This is the first time that formation of a highly reduced volatile metal species (stibine or arsine (AsH_3)) by *C.humicolus* has been described. Arsine production by *C.humicolus* has not been previously reported in the literature, although the volatilisation of trimethylarsine as sole volatile arsenic species from sodium arsenate, sodium arsenite and various organoarsenic compounds has been previously described,[13, 42, 43]. It should be noted however that Cheng and Focht [30] reported that the column used by Cox and Alexander [13] was unsuitable for the separation of arsines, and that the identification of trimethylarsine may therefore be erroneous, or that other arsine species may be present.

The volatilisation of stibine from inorganic antimony substrate has been described previously for another fungus *Scopulariopsis brevicaulis*. Andrewes *et al.* [10] describe the detection of trace levels of stibine, monomethylstibine and dimethylstibine in incubations of the fungus amended with potassium antimony tartrate and isotopically enriched (98.7%) ^{123}potassium hexahydroxyantimonate. In addition, they state that no significant change in the isotopic ratio of antimony in volatile antimony species was observed and concluded that no volatilisation of antimony V substrate occurred. It is possible however that volatilisation may have occurred, but was at a much reduced level to antimony III volatilisation, such that isotopic ratio was not greatly affected.

The observation that stibine or arsine production was higher when inorganic antimony or arsenic was supplied in the V valency state to *C.humicolus* incubations may suggest that this organism possesses the ability to oxidise antimony (or arsenic) III, and subsequently reduce the product species to the volatile stibine (or arsine). Andrewes *et al.* [10] mentioned the ability of the fungus *S.brevicaulis* to oxidise antimony III to antimony V. As yet, however, the only definitive report of antimony bio-oxidation is that of the bacteria *Stibiobacter senarmontii* [44] which derives energy for growth from the oxidation of antimony trioxide.

Data regarding trivalent antimony concentration of potassium hexahydroxyantimonate substrate was not available. However the supposition that the stibine (SbH_3) detected in culture headspace gases of incubations supplied with potassium hexahydroxyantimonate is from low level contaminating trivalent antimony is difficult to reconcile with the observation of the absence of stibine in culture headspace gases of incubations supplied with trivalent antimony substrate.

It should also be considered that the experimental design used here (i.e. ten-fold concentrated biomass, aerobic / anaerobic biphasic incubation) for the detection of volatile antimony and arsenic species, whilst increasing the probability that species susceptible to oxidation will be preserved, may also place the cells under great physiological stress. The possibility that arsine is produced under such conditions is not discounted, however the maximum total amount of volatile arsenic compounds detected (28.6 ng in arsenic V incubations) in the headspace of biphasically incubated cultures is far short of the total mobilised to the distal HNO_3 traps by completely aerobic incubations, as discussed in section 4.3.3. The comparison of these data suggests that volatilisation of arsenic progresses at a much reduced level when cells are placed under physiological stress.

Methylation of antimony by *C.humicolus* was shown to be a biogenic process, dependant upon protein synthesis. In addition, it was demonstrated that the capability to biomethylate antimony is not a feature of the linear phase of growth (as for biomethylation of antimony by *S.brevicaulis*,[9] but is developed, at least in part, by the mid-stationary phase.

Biomethylation from antimony III compounds (potassium antimony tartrate and antimony trioxide) was greater than from antimony V (potassium hexahydroxyantimonate), both in terms of production of volatile methylantimony species and production of involatile forms. As has been suggested before, [9, 10] this may be a result of a requirement for pre-reduction of antimony V to antimony III before methylation can occur. Alternatively, it may reflect the reduced uptake rate of arsenic V compared to arsenic III, and may also indicate a preferential induction of the biomethylation process by arsenic III compounds. Investigations here also demonstrated

that uptake of antimony III and V compounds is distinctly different; with uptake of potassium hexahydroxyantimonate (antimony V) being completely dependant upon respiratory metabolism and occurring at a reduced rate compared to uptake of antimony III compounds. These findings are inconsistent with the claim of Andrewes *et al.* [10] who suggested that fungi are unable to uptake and subsequently methylate antimony V compounds.

Uptake of antimony trioxide (antimony III) was observed to be respiratory metabolism-independent, whilst that of potassium antimony tartrate (antimony III) was partly dependant upon respiratory metabolism, possibly due to cellular uptake *via* the organic tartrate moiety. However, extensive growth by *C.humicolus* using potassium antimony tartrate or tartaric acid as carbon source did not occur. For arsenic, similar observations regarding higher rates of uptake for arsenic III valency compounds compared to arsenic V were noted. In addition, rates of uptake of arsenic were far higher than those observed for antimony.

The range of involatile methylantimony species detected in culture supernatants was different to that observed for volatile methylantimony species in culture headspace gases; trimethylstibine was the only volatile methylated antimony species detected at any time in culture incubations. In contrast, involatile mono-, di- and trimethylated antimony species were identified in culture supernatants. By comparison to the arsenic methylation pathway described by Challenger,[7] the likely speciation of these compounds is monomethylstibonic acid, dimethylstibinic acid and trimethylantimony oxide. It should be noted however, that this is purely suggestive since structure specific evidence outside of the degree of alkylation of compounds and an extensive range of antimony standards were unavailable. The overall amount of involatile methylantimony species increased throughout the incubation period, but was noted to do so in a biphasic manner. That is to say, a modest rate of formation of methylantimony species during the stationary phase of growth was noted, with the rate of formation increasing significantly during the death phase (an almost ten-fold difference in rates of formation of trimethylated antimony species was observed). Cullen *et al.* [5] also noted significant increases in the rate of formation of involatile trimethylarsenic species from sodium arsenate in *C.humicolus* incubations after 7-8 days incubation.

The increased detection of methylantimony species in culture supernatant of *C.humicolus* incubations in later stages of growth might be an apparent increase resulting from the release of species formed at an earlier stage in the incubation, for example through cell lysis. Examination of lysed cells from 6-day incubations revealed a similar ratio of methylantimony species to that typically observed in culture supernatants of 21-day incubations. The observation of no significant difference (at 95% confidence level) in the rates of production of methylantimony species whether inorganic antimony substrate was presented at 0 or 7-days, demonstrates that whilst the mechanism is not linked to the linear phase of growth, it may in fact be a feature of the stationary or death phase. No literature evidence could be found however, to support the theory of increased biomethylation during the death phase. In particular, the intracellular pool of S-adenosylmethionine, the likely source of donor methyl groups, is known to reduce significantly during the death phase.[45] The deleterious effect of cycloheximide upon production of methylantimony species formation when added at the mid-stationary phase (t = 7 days), and the almost complete absence of methylantimony species when added at the end of the linear growth phase (t = 3 days) indicate that biomethylation of antimony by *C.humicolus* is an enzymatic process. Furthermore, it demonstrates that the capability to biomethylate antimony is not a feature of the linear phase of growth, rather that this capability is developed, at least in part, by the mid-stationary phase. This is distinct from the profile of biomethylating capability of *S.brevicaulis*, the only other fungi for which an methylantimony production profile has been given.[9, 10] Since the cells are no longer actively growing and synthesising macromolcules, the increase in antimony biomethylation during the death phase may suggest that the cells have an excess of NADH and that they are seeking to maintain the catabolic reduction charge at 0.05 by reductively methylating antimony (or arsenic).

The possibility of spontaneous transmethylation of antimony by released biogenic compounds such as methyliodide, or methylsulfide as has been demonstrated for the methylation of tin and lead [46, 47] can be discounted, since if this were to be the case, the rate of formation of monomethylantimony species would be significantly greater than dimethylantimony, or trimethylantimony species. Rates of formation observed were 0.3, 1.9 and 3.5 $ng.ml^{-1}$ per day for monomethylantimony, dimethylantimony and trimethylantimony species respectively.

Trimethylantimony oxide was the predominant involatile methylated species detected in culture supernatants in incubations supplied with 50 $mg.l^{-1}$ potassium antimony tartrate, although mono and dimethylated antimony species were also detected. Levels of all three methylated species were observed to increase throughout the incubation period. Intracellular accumulation of involatile methylated antimony species was observed; up to 100-fold higher than in culture supernatants. Differences were noted in the ratios of involatile methylantimony species located within the cell and found in culture supernatants. For cultures supplied with 50 $mg.l^{-1}$ potassium antimony tartrate, trimethylantimony oxide comprised the majority (up to 89%) of the involatile methylantimony species detected in culture supernatant. Within the cell however, the proportion of trimethylantimony oxide was much lower (52.2%) with dimethylantimony and monomethylantimony species comprising 39.4 and 8% respectively. These data suggest that trimethylantimony oxide is the primary exported species and that very low amounts of the oxide are subsequently reduced to volatile trimethylstibine by the organism. It should also be considered that trimethylstibine is highly susceptible to oxidation and may in fact be the primary exported species, to be subsequently abiotically oxidised.

Since the physico-chemical properties of arsenic and antimony are so similar, it is reasonable to suppose that the Challenger mechanism, which has been described for the biomethylation of arsenic [7](and demonstrated to be applicable in *C.humicolus* incubations supplied with arsenic,[5] will also be applicable to the biomethylation of antimony. The enzymes involved in the biomethylation of arsenic are the most likely catalysts for antimony biomethylation. Detection of volatile trimethylstibine in the headspace of cultures suggests that the fungus is able to take antimony through the entire analogous biochemical pathway described for arsenic. In contrast to *C.humicolus*, no involatile monomethylantimony species were detected in *S.brevicaulis* incubations supplied with 50 $mg.l^{-1}$ potassium antimony tartrate. In addition, the relative proportions of involatile mono- di- and trimethylated species were different to those observed for *C.humicolus*. This suggests that these chemical species are intermediates in the antimony biomethylation pathway, rather than products arising though degradative oxidation of trimethylstibine.

The correlation between total involatile methylantimony species in culture supernatants and the proportion existing as dimethylated antimony species suggests that the further methylation of dimethylstibinic acid is a rate determining step in the antimony biomethylation pathway for both *C.humicolus* and *S.brevicaulis*. Accumulation of involatile dimethylated antimony species during aerobic *S.brevicaulis* incubation has been reported previously and suggested to arise as a result of a rate limiting transformation to trimethylated antimony species.[10] When cells were pre-incubated with arsenic, or arsenic was included in incubations (i.e. in addition to antimony), the rate of formation of trimethylantimony oxide was significantly increased (at 95% confidence level). In addition, no mono- or dimethylated antimony species were observed. These data are consistent with induction of antimony biomethylation by arsenic. However, the methylation of antimony was found not to be stimulated by pre-incubation of cells with potassium antimony tartrate, indicating that inorganic antimony does not induce the enzymes responsible for its methylation. This also suggests that the enzymes involved in the biomethylation of arsenic are the catalysts for antimony biomethylation.

The detection of an unknown antimony peak (t = 2.3 min) when co-incubated levels of arsenic were elevated is interesting since Grüter *et al.* [48] reported low levels of one or possibly two unknown antimony compounds, with boiling point higher than trimethylstibine, in hydride generated soil samples from a municipal waste deposit. The identity of these compounds was not speculated upon by these workers. It is worth considering that compound X-Sb may be a metabolised product of trimethylstibine, however no correlation between high trimethylstibine levels and the presence of compound X-Sb was observed. X-Sb could not only be independent of trimethylstibine concentration, but may even be formed by an alternative, arsenic induced (?), mechanism. Whilst the amount of X-Sb detected in culture supernatants increased when arsenic V levels were increased from 50 $mg.l^{-1}$ to 500 $mg.l^{-1}$ (6% of antimony species detected at As = 50 $mg.l^{-1}$, increasing to 27% when As=500 $mg.l^{-1}$), no trend can be inferred since these are only two data points. The suggestion that the formation of X-Sb is arsenic induced is therefore tentative. The possibility that the peak may be dimerised dimethylstibine, that is tetramethyldistibine ($Me_2SbSbMe_2$) can be discounted on the basis of boiling point; unknown peak bp= 89.5°C, tetramethyldistibine = 53°C.[49] In addition, the compound tetramethylsdistibine is highly susceptible to cleavage of the Sb-Sb bond by oxidation. In an aerobic environment such as achieved in *C.humicolus*

incubations tetramethyldistibine would most probably be oxidised to dimethylstibinic acid.

Comparison of methylation efficiencies from arsenic and antimony substrates by *C.humicolus* revealed that biomethylation of arsenic is significantly (up to 100-fold) more favourable than antimony biomethylation. Less than 10% of inorganic arsenic substrate was methylated compared to less than 0.1% of inorganic antimony substrate. Efficiencies for the biomethylation of antimony and arsenic by *S.brevicaulis* reveal that this organism too favours arsenic biomethylation over that of antimony by 1463-fold.[10, 32]

Since trimethylantimony oxide was shown not to associate with biomass through external surface binding or to be taken up by the cell, passively or actively, it is proposed that the extracellular transport of trimethylantimony oxide must be a selective and active process, rather than occurring by passive diffusion as was suggested by Andrewes *et al*..[10] The movement of dimethylstibinic acid to culture media may be occurring by passive diffusion, or may be a substrate induced active transport mechanism operational once intracellular concentration of dimethylstibinic acid is sufficiently high. It is difficult to distinguish between the two processes in the absence of dimethylated antimony standards for surface/uptake binding studies.

Differences in rates of uptake between arsenic and antimony and the valency III and V forms, explain in part the differences observed in rates of methylation of arsenic and antimony compounds. Other factors must also impact however, since whilst biomethylation of antimony is 100-fold less than biomethylation of arsenic (based on extracellular levels of methyl species), its association with *C.humicolus* biomass is only 2-fold less. The question must therefore be, "What happens to the rest of the antimony that is taken up by the cell?" Intracellular levels of methylantimony species are not sufficient to account for the "missing antimony" (amounting to 36.19 $\mu g.g^{-1}$ biomass after 6-days incubation compared to a total association of 259.2 $\mu g.g^{-1}$ biomass over this period). Likewise whilst volatile species are produced by *C.humicolus* (both stibine and trimethylstibine were detected) the formation of these is too slight to account for the "missing antimony". Despite a slowing in the rate of association of antimony, uptake was still favoured over export, indicating that intracellular accumulation most probably

occurs. Analysis of non-alumina cleaned cell supernatants from incubations supplied with potassium antimony tartrate by HPLC-hydride generation-AFS did not reveal the presence of peaks additional to the three methyl peaks usually observed. Whilst at first sight this result would appear to indicate that the stored antimony is in the III valency form, since antimony III compounds are not eluted from the column, it should also be considered that the absence of further peaks would be observed for antimony V compounds if they could not be hydride generated, or were retained themselves on the column.

It is possible that the "missing antimony" is associated with biomass in an inorganic III form removed from samples prior to analysis by the alumina column treatment, or that the antimony is present in a non-hydride generatable organo-antimony form. The possibility of biosorption of transformed or non-transformed antimony (removed by centrifugation treatment prior to analysis) can also not be discounted.

Regarding speciation of any exported inorganic antimony species, HPLC-hydride generation-AFS analysis of non-alumina cleaned supernatants did not reveal the presence of additional antimony containing peaks. This suggests that the exported antimony species are in the III valency form since these are retained by the column under the conditions used. The possibility of the presence of V valency species that are not eluted from the column, or that can not be hydride generated can not however be discounted. Hydride generation-GC-AAS analysis of inorganic antimony species results in the formation of stibine alone and does not therefore allow for speciation. This was not therefore pursued.

The occurrence of a carrier saturation effect allows distinguishment between the two forms of diffusion, namely passive (for which a linear transportation gradient is obtained) and facilitated (for which an exponential transportation curve due to saturation of transport proteins is obtained). The apparent decrease in the rate of association of inorganic antimony species of either valency by *C.humicolus* after 18 hours incubation may be explained by considering that carrier saturation of uptake proteins [active transport (antimony V) and facilitated diffusion (antimony III)] is occurring. Continued uptake of antimony III by facilitated diffusion is maintained by

biotransformation of the species to an alternative form (e.g. methylantimony species), and/or extracellular export.

No change in the rate of trimethylarsine oxide association with biomass was noted, indicating that this species, or its product, is not subsequently transported to the liquid culture media. Pickett *et al.* [43] reported a rate of volatilisation of 0.12 μg trimethylarsine per g biomass per minute from trimethylarsine oxide substrate, above the rate of metal-biomass association of 0.09 μg trimethylarsine oxide per g biomass per minute observed here. These data taken together may indicate that all of the trimethylarsine oxide associated (excluding that which is surface-bound) may be volatilised to trimethylarsine. Cullen *et al.* [32] reported that the molcule trimethylarsine oxide is capable of passive diffusion from *C.humicolus* cells to culture media. Clearly extracellular levels of trimethylarsine oxide exceed those within the cell in this experiment hence extracellular passive diffusion does not occur. The continued uptake of trimethylarsine oxide is likely maintained by its intracellular transformation and volatilisation to trimethylarsine.

The observation that antimony III is more toxic than the V valency form, and that arsenic in either valency is more toxic than antimony may result from differences in uptake of the various species, in addition to differences in site of cellular action. As mentioned before, little is known about the cellular mechanisms of toxicity for the various antimony species, and it is often conjectured that antimony and arsenic will display similar toxicological properties.[25, 26, 50] With regard to the lower oxidation state being the more toxic, this is certainly the case. Antimony III compounds, as for arsenic III, are known to possess a predilection for associating with sulfhydryl group, interfering with enzyme activity. Little evidence exists, however, as to the formation of antimonylated sugars or other interchanges with phosphate that are the basis of arsenic V toxicity.

The donation of methyl groups by L-methionine-*methyl-d_3* has previously been described in *C.humicolus* incubations (to the metal arsenic)[14, 32], and to the metalloid antimony (in *S.brevicaulis* incubations).[27] These reports suggest that L-methionine-*methyl-d_3* would also be able to serve as methyl donor for the biomethylation of antimony by *C.humicolus*. The lack of detection of deuterated methylantimony species

in this investigation may not necessarily imply absence of *methyl-d_3* transfer from L-methionine-methyl-d_3, but may be a factor of working at the limits of sensitivity of this technique. The total amount of trimethylstibine detected in non-labelled incubations by GC-MS was at the absolute detection limit for this species (0.49 μg). If *methyl-d_3* groups were incorporated to trimethylstibine in labelled incubations, the total amount of labelled trimethylantimony species would comprise a proportion of the whole and would therefore be below the absolute detection limit of this technique.

Within cellular metabolism, the relationship between the three co-enzymes S-adenosylmethionine, N-tetrahydrofolate, and methylcobalamin is complex and interdependent. Whilst S-adenosylmethionine is involved in the vast majority of transmethylation reactions within a cellular environment and so could be the likely candidate; the possibility that alternative methyl donors such as methylcobalamin and N-methyltetrahydrofolate may be capable of the biomethylation of antimony by *C.humicolus* cannot be immediately discounted. Methylation of the metalloid antimony requires transfer of a carbocationic methyl group, i.e. CH_3^+.[2] Theoretically, all three of the major co-enzymes are capable of methyl group transfer in this way. Methylcobalamin moreover is the only one of the three that is additionally capable of anionic and radical methyl group transfer, i.e. CH_3^- and $CH_3\bullet$.[51] Examination of tetrahydrofolate and methylcobalamin in *in vivo* systems is however problematic. Labelled methyl groups present on these two coenzymes will also find their way to S-adenosylmethionine *via* the activated methyl cycle (Figure 4.16). This argument does not however operate in reverse. Cullen *et al.* [32] reasoned that the transfer of *methyl-d_3* groups from L-methionine-*methyl-d_3* to antimony demonstrates that S-adenosylmethionine is the biological methyl donor, since L-methionine is a precursor of S-adenosylmethionine. *In vivo* investigations of S-adenosylmethionine as methyl donor in microorganisms must however be run in parallel with *in vitro* studies to ascertain that the coenzyme is the direct methyl donor to arsenic and does not operate one or two steps removed. That is to say, S-adenosylmethionine methylates some other compound, which in turn serves as methyl donor to antimony. This has been shown to be the case for the biomethylation of arsenic in *Methanobacterium*, where the molcule coenzyme-M serves as actual methyl donor to the metal, sourcing its methyl groups from the supplied methylcobalamin.

The biomethylation of inorganic arsenic in mammals is catalysed by arsenic methyltransferases requiring glutathione (GSH) and S-adenosylmethionine as essential cofactors,[52, 53, 54]. S-adenosylmethionine serves as methyl group donor, whilst glutathione is essential for the protection of labile thiol groups of arsenic methyltransferases [54]. Additionally, the redox cycling of glutathione may be involved in the reduction of arsenic V compounds to the III valency after the addition of a methyl group.[55, 56] It seems reasonable to postulate that S-adenosylmethionine and glutathione will be the essential cofactors required for arsenic (and antimony) biomethylation in the eukaryotic yeast and fungi. It is interesting to note that the thiol groups of glutathione do not appear themselves to be directly involved in arsenic methylation, but protect some other thiol source. Buchet and Lauwerys [57] and Georis *et al.* [58]demonstrated that reduction of *in vivo* glutathione levels in test animals caused a reduction in arsenic methylation in liver cytosol (tested *in vitro*). Subsequent restoration of liver cytosol levels did not restore arsenic methylation capability, indicating that other labile thiols are critical for the methylation of arsenic and that these thiols are normally protected by glutathione.

As yet, the only study to investigate the effect of a co-enzyme (as opposed to a pre-cursor) on microbial methylation of these metals was by Cullen *et al.*.[59] The group reported no methylarsenic incorporation of the ^{14}C-methyl moiety from S-adenosylmethionine-^{14}C-methyl in cell extracts. These authors also reported that inclusion of a range of electron transport inhibitors and methylating agents such as S-adenosylmethionine in cell free extracts, produced no effect on the yield of dimethylated arsenic from monomethylated arsenic substrate. The same group later demonstrated however, the importance of the electron transport chain in generation of reducing conditions for the last stage of arsenic methylation, that is the reduction of trimethylarsenic oxide to trimethylarsine, concluding that it is necessary to have a "biologically intact organism capable of generating appropriate reducing conditions".[59]

In conclusion, the biomethylation of various inorganic antimony substrates by *C.humicolus* has been shown. This was demonstrated to be an enzymatic process, inducible by arsenic III or V, but not by antimony. Trimethylantimony oxide is the primary exported species to culture media, with monomethylstibonic acid and dimethylstibinic acid serving as likely precursors. The transformation step from di- to

trimethylated antimony appears to be rate limiting since the supernatant concentration of dimethylated antimony species is proportional to the total concentration of methylated species. The similarities between the processing of arsenic and antimony by *C.humicolus* are marked. Coupled with the induction of antimony biomethylation by arsenic, these data strongly suggest that not only is the biomethylation of antimony is a fortuitous process; it is performed by the enzymes responsible for arsenic methylation. It is likely that the formation of stibine and any non-methylated involatile species is also performed by arsenic substrate enzymes. This remains to be established however. A suggested outline for the processing of antimony by *C.humicolus* is shown in Figure 4.17.

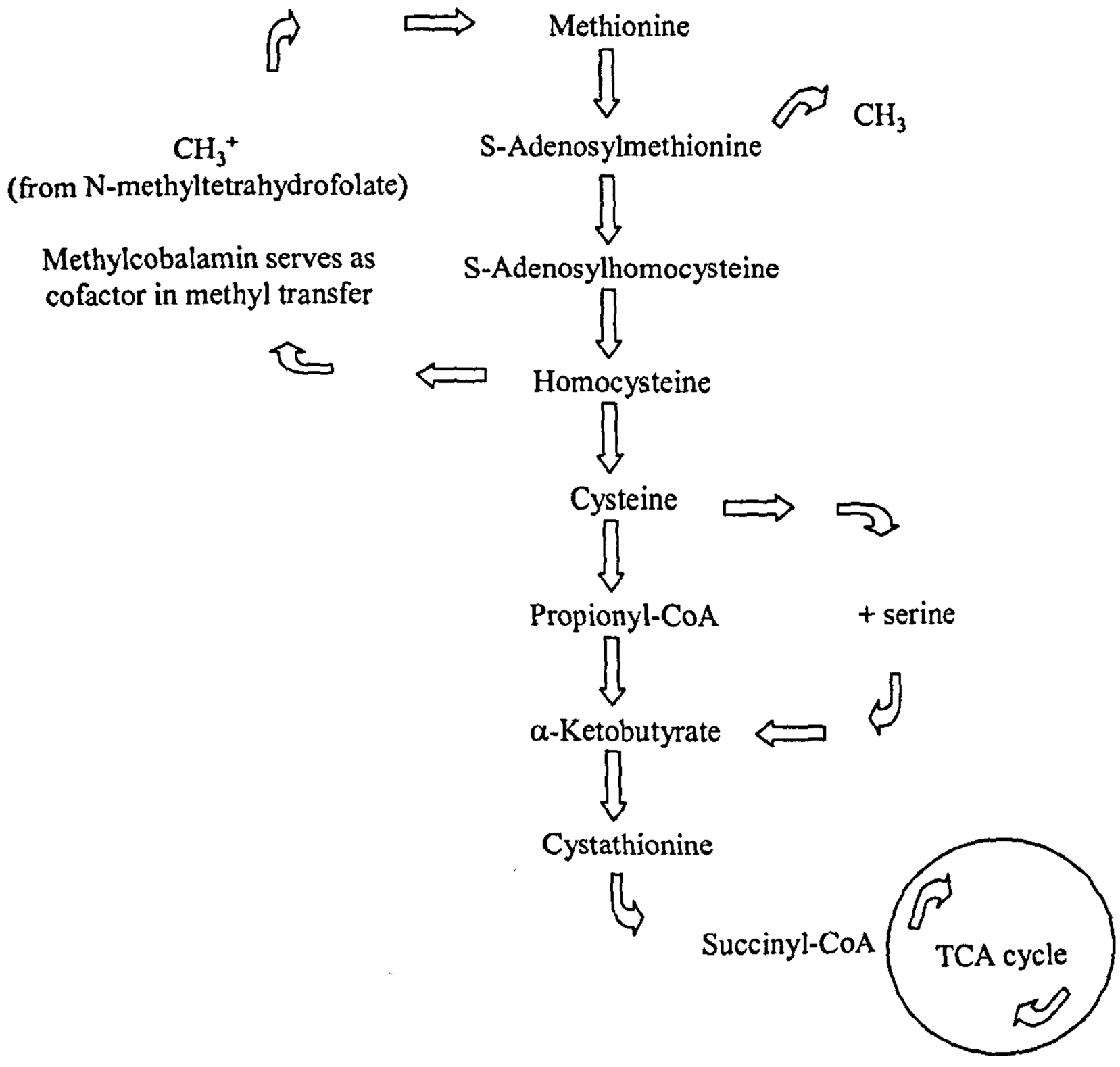


Figure 4.16 Activated methyl cycle.

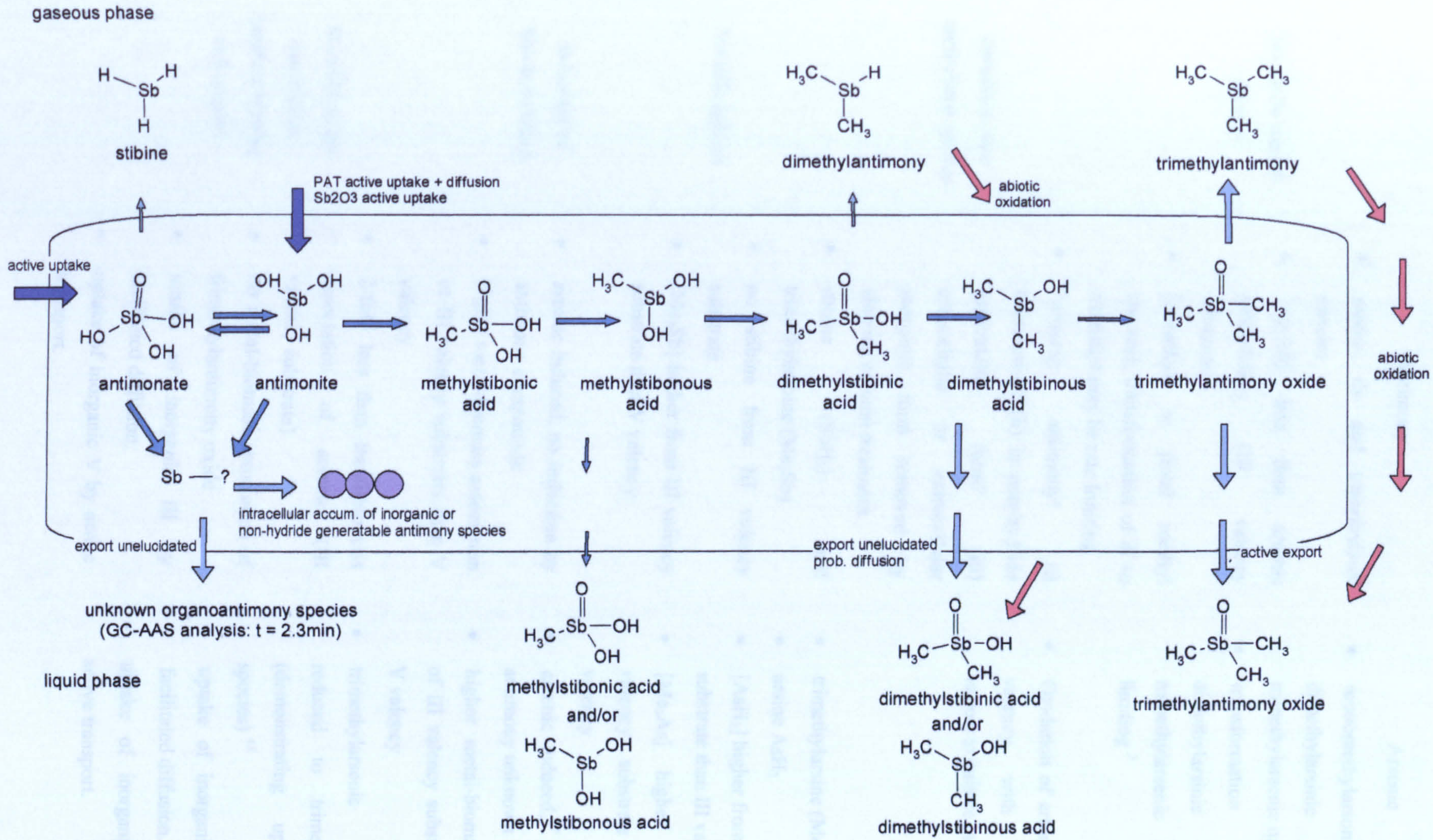


Figure 4.17 Proposed processing of antimony by *Cryptococcus humicolus*. Based in part upon the Challenger mechanism for the methylation of arsenic.

4.5 Summary; comparison of antimony and arsenic processing by *C.humicolus*

	Antimony	Arsenic
Involatile methyl species	• mono-, di- and trimethylated species • 100-fold less than arsenic methylation (III valency substrate) • [dimethyl] ∝ [total methyl species]; transformation of di to trimethyl may be rate limiting	• monomethylarsonic acid, dimethylarsinic acid, trimethylarsenic oxide [5] • transformation of dimethylarsinic acid to trimethylarsenic oxide rate limiting [5]
Involatile non-methylated species	• "missing antimony" (i) biosorption? (ii) in non-hydride generatable form? (iii) intracellular or extracellular inorganic form removed by alumina column treatment.	• Oxidation of arsenic V to III valency with subsequent export to culture media [5]
Volatile species	• stibine (SbH_3) and trimethylstibine (Me_3Sb) • no stibine from III valency substrate • [Me_3Sb] higher from III valency substrate than V valency	• trimethylarsine (Me_3As) [13] • arsine AsH_3 • [AsH_3] higher from V valency substrate than III valency • [Me_3As] higher from III valency substrate than V valency
Induction of biomethylation	• arsenic induced, no induction by antimony compounds • higher metal-biomass association of III valency substrates than V valency	• arsenic induced,[42] impact of antimony unknown • higher metal-biomass assoc. of III valency substrates than V valency
Metal-biomass association (surface binding and uptake)	• 2-fold less than metal-biomass association of arsenic (III valency substrate) • no metal-biomass association of trimethylantimony oxide • uptake of inorganic III by facilitated diffusion. • uptake of inorganic V by active transport.	• trimethylarsenic oxide reduced to trimethylarsine (demonstrating uptake of species) [43] • uptake of inorganic III by facilitated diffusion. • uptake of inorganic V by active transport.

Toxicity (resistance)	• Toxicity of III valency substrates higher than V valency • III valency substrates – sulfhydryl bonding [26] • V valency substrates – unknown action	• Toxicity of III valency substrates higher than V valency [60] • III valency substrates – sulfhydryl bonding [39] • V valency substrates – phosphate displacement [39]
Source of methyl groups	• Probably S-adenosylmethionine (unproved)	• S-adenosylmethionine [32]

References for Chapter 4

1. W. R. Cullen and K. J. Reimer, Arsenic speciation in the environment. *Chemical Reviews* **89**, 713 (1989).
2. O. S. Fatoki, Biomethylation in the natural environment: A review. *South African Journal of Science* **93**, 366 (1997).
3. J. S. Thayer and F. E. Brinckman, The biological methylation of metals and metalloids. *Advances in Organometallic Chemistry* **20**, 313 (1982).
4. F. Challenger, C. Higginbottom, and L. Ellis, The formation of organometalloidal compounds by microorganisms. Part 1: Trimethylarsine. *Journal of the Chemical Society* 95 (1933).
5. W. R. Cullen, H. Li, G. Hewitt, and K. J. Reimer, Identification of extracellular arsenical metabolites in the growth medium of the microorganisms *Apiotrichum humicola* and *Scopulariopsis brevicaulis*. *Applied Organometallic Chemistry* **8**, 303 (1994).
6. D. Barnard, The attempted methylation of compounds of antimony, mercury, tin and lead by *Scopulariopsis brevicaulis* and *Penicillium notatum*. 9. University of Leeds, U.K., Leeds, (1947).
7. F. Challenger, Biological methylation. *Chemical Reviews* **36**, 315 (1945).
8. D. K. Barrett, An improved selective medium for isolation of *Phaeolus schweinitzii*. *Transactions of the British Mycological Society* **71**, 507 (1978).
9. R. O. Jenkins, P. J. Craig, W. Goessler, D. Miller, N. Ostah, and K. J. Irgolic, Biomethylation of inorganic antimony compounds by an aerobic fungus: *Scopulariopsis brevicaulis*. *Environmental Science and Technology* **32**, 882 (1998).
10. P. Andrewes, W. R. Cullen, J. Feldmann, I. Koch, P. E., and K. J. Reimer, The production of methylated organoantimony compounds by *Scopulariopsis brevicaulis*. *Applied Organometallic Chemistry* **12**, 827 (1998).
11. Thom, and Raper, *in* F. Challenger, Biological Methylation. *Chemical Reviews* **36**, 315 (1945).
12. M. L. Bird, F. Challenger, P. T. Charlton, S. J. O., *in* W. R. Cullen, and K. J. Reimer, Arsenic speciation in the environment. *Chemical Reviews* **89**, 713 (1989).
13. D. P. Cox and M. Alexander, Production of trimethylarsine gas from various arsenic compounds by three sewage fungi. *Bulletin of Environmental Contamination & Toxicology* **9**, 84 (1973).
14. W. R. Cullen, C. L. Froese, A. Lui, M. B. C., D. J. Patmore, and M. Reimer, The aerobic methylation of arsenic by microorganisms in the presence of L-methionine-*methyl-*d_3. *Applied Organometallic Chemistry* **139**, 62 (1977).
15. F. V. Vidal and V. M. V. Vidal, Arsenic metabolism in marine bacteria and yeast. *Marine Biology* **60**, 1 (1980).
16. Verona, *in* F. Challenger, Biological Methylation. *Chemical Reviews* **36**, 315 (1945).
17. R. A. Zussman, E. E. Vicher, and I. Lyon, Arsine production by *Trichophyton rubrum*. *Journal of Bacterialogy* **81**, 157 (1961).
18. B. Gosio, Zur Frage, wodurch die Gifitgkeit arsenhaltiger Tapeten bedingt wird. *Berichte der Deutschen Chemischen Gesellschaft* **30**, 1024 (1897).
19. Pool , *in* F. Challenger, Biological Methylation. *Chemical Reviews* **36**, 315 (1945).

20. F. Challenger, *in* Cullen, W. R. and K. J. Reimer, Arsenic speciation in the environment. *Chemical Reviews* **89**, 713 (1989).

21. K. D. Huysmans and W. T. Frankenberger Jnr., Arsenic resistant microorganisms isolated from agricultural drainage water and evaporation pond sediments. *Water, Air, and Soil Pollution* **53**, 159 (1990).

22. M. Shariatpanahi, A. C. Anderson, and A. A. Abdelghani, *in* Trace substances in environmental health D. D. E. *in* Hemphill, Vol. Part X, p. 383. University of Missouri, Columbia, 1981.

23. RSC, Chemical safety data sheets. Volume 2. Main group metals and their compounds. Royal Society of Chemistry, London, (1989).

24. L. Friberg, G. F. Nordberg, and V. B. Vouk, Handbook on the toxicology of metals. Volume II: Specific metals. *Elsevier Science Publisher B.V.* 27 (1986).

25. A. Leonard and G. B. Gerber, Mutagenicity, carcinogenicity and teratogenicity of antimony compounds. *Mutation Research* **366**, 1 (1996).

26. K. A. Winship, Toxicity of antimony and its compounds. *Adverse Drug Reaction and Acute Poisoning Reviews* **2**, 67 (1987).

27. P. Andrewes, W. R. Cullen, J. Feldmann, I. Koch, and E. Polishchuk, Methylantimony compound formation in the medium of *Scopulariopsis brevicaulis* cultures: $^{13}CD_3$-L-methionine as a source of the methyl group. *Applied Organometallic Chemistry* **13**, 681 (1999).

28. B. A. Richardson, Sudden Infant Death Syndrome: a possible primary cause. *Journal of the Forensic Science Society* **34**, 199 (1994).

29. K. Michalke, E. B. Wickenheiser, M. Mehring, A. V. Hirner, and R. Hensel, Production of volatile derivatives of metal(loid)s by microflora involved in anaerobic digestion of sewage sludge. *Applied and Environmental Microbiology* **66**, 2791 (2000).

30. C.-N. Cheng and D. D. Focht, Production of arsine and methylarsines in soil and in culture. *Applied and Environmental Microbiology* **38**, 494 (1979).

31. P. Andrewes, W. R. Cullen, and E. Polishchuk, Arsenic and antimony biomethylation by *S.brevicaulis*: Interactions of arsenic and antimony compounds. *Environmental Science and Technology* **34**, 2249 (2000).

32. W. R. Cullen, H. Li, S. A. Pergantis, G. K. Eigendorf, and A. A. Mosi, Arsenic biomethylation by the microorganism *Apiotrichum humicola* in the presence of L-methionine-*methyl-d_3*. *Applied Organometallic Chemistry* **9**, 507 (1995).

33. R. Yamada, H. Ujiie, Y. Kera, T. Nakase, K. Kitagawa, T. Imasaka, K. Arimoto, N. Takahashi, and Y. Matsumura, Purification and properties of D-aspartate oxidase from *Cryptococcus humicolus UJ1*. *Biochimica et Biophysicts Acta* **1294**, 153 (1996).

34. Sigma-Aldrich, Certificate of Analysis. (2001).

35. J. Lintschinger, I. Koch, S. Serves, J. Feldmann, and W. R. Cullen, Determination of antimony species with high-performance liquid chromatography using element specific detection. *Fresenius Journal of Analytical Chemistry* **359**, 484 (1997).

36. W. J. Middelhoven, M. Koorevaar, and G. W. Schuur, Degradation of benzene compounds by yeast in acidic soils. *Plant and Soil* **145**, 37 (1992).

37. P. A. E. P. Meesters, G. N. M. Huijberts, and G. Eggink, High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. *Applied Microbiology and Biotechnology* **45**, 575 (1996).

38. E. C. Chang, D. J. Kosman, and G. R. Willsky, Arsenic oxide-induced thermotolerance in *Saccharomyces cerevisiae. Journal of Bacteriology* **171**, 6349 (1989).

39. S. A. Lerman, T. W. Clarkson, and R. J. Gerson, Arsenic uptake and metabolism by liver cells is dependent on arsenic oxidation state. *Chemico-Biological Interactions* **45**, 401 (1983).

40. D. P. Cox, M. C. Alexander, *in* W. R., and K. J. Reimer, Arsenic speciation in the environment. *Chemical Reviews* **89**, 713 (1989).

41. K. D. Spence, A. J. Ferro, and T. F. Petrotta-Simpson, *in* The Biochemistry of Adenosylmethionine F. Salvatore, E. Borek, V. Zappia, H. G. Williams-Ashamn, and F. Schlenk, Vol. 77. Columbia University Press, New York, 1977.

42. W. R. Cullen, B. C. McBride, and M. Reimer, Induction of the aerobic methylation of arsenic by *Candida humicola. Bulletin of Environmental Contamination and Toxicology* **21**, 157 (1979).

43. A. W. Pickett, B. C. McBride, W. R. Cullen, and H. Manji, The reduction of trimethylarsine oxide by *Candida humicola. Canadian Journal of Microbiology* **27**, 773 (1981).

44. N. N. Lyalikova, Oxidation of trivalent antimony up to the highest oxides as a source of energy for the development of the new autotrophic organism Stibiobacter Gen. N. *Mikrobiologika* **205**, 1228 (1972).

45. D. Sinclair, K. Mills, and L. Guarente, Aging in *Saccharomyces cerevisiae. Annual Review of Microbiology* **52**, 533 (1998).

46. A. W. P. Jarvie and A. D. Whitmore, Methylation of elemental lead and lead (II) salts in aqueous solutions. *Environmental Technology Letters* **2**, 197 (1981).

47. S. Rapsomanikis and J. H. Weber, Environmental implications of methylation of Sn(II) and methyltin(IV) ions in the presence of manganese dioxide. *Environmental Science and Technology* **19**, 352 (1985).

48. U. M. Grüter, J. Kresimon, and A. V. Hirner, A new HG/LT-GC/ICP-MS multi-element speciation technique for real samples in different matrices. *Fresenius Journal of Analytical Chemistry* **368**, 67 (2000).

49. A. B. Burg, *Journal of the American Chemical Society* **81**, 1 (1959).

50. L. Friberg and C. G. Elinder, Biological monitoring of toxic metals. *Scandinavian Journal of Work and Environmental Health* **19**, 7 (1993).

51. J. M. Wood, Biological methylation of mercury can occur by many routes, says biochemist Wood. *Chemistry and Engineering News* 24 (1971).

52. J. P. Buchet and R. Lauwerys, Study of inorganic arsenic methylation by rat liver in vivo: Relevance for the interpretation of observations in man. *Archives of Toxicology* **57**, 125 (1985).

53. R. Zakharyan, Y. Wu, G. M. Bogdan, and H. V. Aposhian, Enzymatic methylation of arsenic compounds: Assay, partial purification, and properties of arsenite methyltransferase and monomethyltransferase of rabbit liver. *Chemical Research in Toxicology* **8**, 1029 (1995).

54. M. Styblo, M. Delnomdedieu, and D. Thomas, Mono- and dimethylation of arsenic in rat liver cytosol *in vitro. Chemico-Biological Interactions* **99**, 147 (1996).

55. W. R. Cullen, B. C. McBride, and J. Reglinski, The reduction of trimethylarsine oxide to trimethylarsine by thiols: A mechanistic model for the biological reduction of arsenicals. *Journal of Inorganic Biochemistry* **21**, 45 (1984).

56. D. J. Thompson, A chemical hypothesis for arsenic methylation in mammals. *Chemico-Biological Interactions* **88**, 89 (1993).

57. J. P. Buchet and R. R. Lauwerys, Role of thiols in the *in vitro* methylation of inorganic arsenic by rat liver cytosol. *Biochemical Pharmacology* **37**, 3149 (1988).

58. B. Georis, A. Cardenas, J. P. Buchet, and R. R. Lauwerys, Inorganic arsenic methylation by rat liver slices. *Toxicology* **63**, 7384 (1990).

59. W. R. Cullen, B. C. McBride, H. Manji, A. W. Pickett, and A. W. Reglinski, The metabolism of methylarsine oxide and sulfide. *Applied Organometallic Chemistry* **3**, 71 (1989).

60. F. Bertolero, G. Pozzi, E. Sabbioni, and U. Saffiotti, Cellular uptake and metabolic reduction of pentavalent to trivalent arsenic as determinants of cytotoxicity and morphological transformation. *Carcinogenesis* **8**, 803 (1987).

5 BIOTRANSFORMATIONS OF ANTIMONY AND ARSENIC BY OTHER FUNGI

5.1 Introduction

The most important criteria for a high biomethylating potential have been detailed by Hirner *et al.* [1] as; (i) An anaerobic atmosphere (at least on a microscale), reducing conditions and slightly acidic pH values in the hydrosphere; (ii) High concentrations of metal(loid)s in easily accessible forms (e.g. as ions); (iii) The presence of microorganisms with biomethylation potential (bacteria, fungi); (iv) The presence of transferable methyl groups. With regard to point (iii), the range of bacteria known to be capable of antimony biomethylation has been extended in recent times. Mixed inoculum cultures of fermentative, denitrifying and methanogenic bacteria have all been demonstrated to possess a biomethylating capability,[2-4] and pure cultures of three methanogenic archaea species, of *Clostridium collagenovorans* and of *Desulfovibrio vulgaris* have been demonstrated to volatilise trimethylstibine from inorganic antimony substrate.[5] Chapter 3 further extended the range of pure cultures of bacteria known to methylate antimony; *Clostridium acetobutylicum*, *Clostridium butylicum* and *Clostridium cochlearium* were all shown to possess this capability. These reports demonstrate that the ability to biomethylate antimony is not confined to a single genus or metabolic type. The possibility of a similar scenario with regard to antimony biomethylation by fungi is likely. As yet, the only fungi reported as possessing an antimony biomethylating capability are *Scopulariopsis brevicaulis* [6, 7] and *Phaeolus schweinitzii*.[8] The ability of C*ryptococcus humicolus* to methylate inorganic antimony substrate was demonstrated and elucidated in Chapter 4. All of these fungi are known arsenic methylators.

When the range of fungi known to methylate arsenic is considered, it can be seen that the vast majority of these organisms are hyphomycetous anamorphs. The detection of methylarsenic species in a number of basidomycetes has been reported.[9-11] The only report of arsenic biomethylation from a non-hyphomycetous fungus is that of Verona [12]who described the identification of *Saccharomyces ellipsoideus* as an arsenic methylator. This finding was however disputed by Challenger in his 1945 treatise on biological methylation.[13] In light of these reports, it could be suggested that the ability of fungi to biomethylate arsenic or antimony is somehow linked to hyphae formation.

For example, Jenkins *et al.* [14] demonstrated a reduced capability by *S.brevicaulis* to volatilise inorganic antimony when the fungus was in a morphological transition phase to the yeast form. Conversely, the ability of this fungus to volatilise trimethylarsine was reported to be enhanced when it is in the yeast phase [15].

Regarding the taxonomy of organisms studied with regard to a biomethylating capability, *Candida* and *Geotrichum* are closely related to the known arsenic (and antimony) methylator *C.humicolus*. All these organisms are members of the taxonomic order of *Cryptococcaceae*. *C.humicolus* is the only *Cryptococcaceae* shown previously to have methylated arsenic or antimony.[16, 17] *Rhodotorula rubra* is the sole member of the family Rhodotorulaceae. It has been demonstrated to possess the capability to volatilise arsenic with the formation of an unidentified arsine, and is a known arsenate reducer.[18]

S.brevicaulis is a known arsenic methylator and is a member of the family *Aspergilleae*. Other members of this family that have been demonstrated to methylate inorganic arsenic are *Aspergillus*, *Penicillium* and *Gliocladium*.[13, 16, 19, 20] *S.brevicaulis* has recently been demonstrated to methylate antimony, both volatile species and involatile have been identified.[6, 7] Furthermore, the co-enzyme S-adenosylmethionine has been demonstrated to be the source of methyl groups involved in the methylation of both metals.[17, 21] This organism was therefore used as a reference culture.

Saccharomyces cerevisiae is a member of the family *Saccharomycetaceae*. This is the only non-anamorphic fungus that was tested for a biotransformation capability with regard to ability to biotransform antimony or arsenic. As mentioned previously, *S.ellipsoideus* has been reported to possess a capability to methylate arsenic,[12] this however has been disputed.[13] *S.cerevisiae* has previously been demonstrated to possess the capability to methylate mercury from mercuric chloride when growing under anaerobic conditions.[22]

Many organometallic compounds involving elements in the environment are thought to be formed *in situ*.[23-25] As yet, no reports of antimony methylation of volatilisation by aerobic cultures of bacteria exist. This coupled with the fact that fungi are well known to possess the capability to grow utilising carbon from a wide range of sources and

generally display a higher resistance to metals, could indicate that fungal biotransformation of antimony is more significant in environmental terms than that of bacteria.

5.2 Methodology

5.2.1 Organisms and culture conditions

Candida boidinii, *Candida tropicalis*, *Geotrichum candidum*, *Rhodotorula rubra* NCPF 3133, *Saccharomyces cerevisiae*, and *Scopulariopsis brevicaulis* IMI 17297 were all obtained from the Microbiology Culture Collection of De Montfort University, Leicester. All species were maintained by routine subculture on slopes of malt extract agar (Oxoid, Unipath Ltd., Basingstoke, UK). Malt extract broth (Oxoid) was used throughout as cultivation media for these organisms.

Malt extract broth (Oxoid) was prepared such that the final volume occupied 40% of total flask volume, e.g. for a 500 ml flask, 200 ml of medium was required. Culture inoculum of *S.brevicaulis* was prepared from agar streak plates. The surface of the agar plate was flooded with malt extract broth and gently swabbed to suspend spores. The resulting spore suspension was inoculated to culture media such that a final concentration of $2\text{-}3\text{x}10^{5}$ spores.ml^{-1} was obtained. Culture inocula of all other species were prepared from agar streak plates incubated overnight at 28°C. Culture was swabbed off agar plates and resuspended in 10 ml aliquots of fresh liquid medium to produce a turbid cell suspension with an absorbance of 1 unit (λ = 600 nm). Inoculum was inoculated to culture medium at 0.5 ml inoculum per 100 ml. Cultures were incubated at 28°C and 100 rpm in a Gallenkamp orbital incubator for periods of up to 28 days. Where appropriate, control incubations were prepared omitting inoculation of biomass. Biphasic (aerobic / anaerobic) culture incubations were prepared using malt extract broth as described in section 4.2.12.

5.2.2 Additions of metal substrate to culture incubations

Additions of antimony and arsenic as biotransformation substrates were made to culture incubations as detailed in section 4.2.2. Control incubations were prepared without metal addition. Unless otherwise stated, upon sampling, biomass was separated from supernatant by centrifugation, 10 minutes, 4000 rpm. Supernatants were subsequently passed through a basic alumina column 10 ml bed volume; methylantimony species were eluted from the column using 0.1 mol.l^{-1} potassium acetate (pH 7.5) at a flow rate of 1 ml.min^{-1}. Supernatants were stored at –20°C until analysis was performed.

5.2.3 Comparison of inhibitory effect of arsenic and antimony on growth of *S.cerevisiae*

Malt extract broth was prepared in double concentration and dispensed in 5 ml aliquots to universal vials. Aliquots of 10 $g.l^{-1}$ potassium antimony tartrate (antimony III), sodium arsenite (arsenic III), or sodium arsenate (arsenic V) were added such that final concentrations of 10, 100, 500, 1000, and 5000 $mg.l^{-1}$ metal in 10 ml total test volume were achieved. Test solutions of potassium hexahydroxyantimonate (antimony V) were prepared in an identical manner from a 5 $g.l^{-1}$ solution. The maximum test concentration for antimony V was 2500 $mg.l^{-1}$ antimony. All universal vials were inoculated with 0.1 ml overnight culture inoculum, resulting in an initial absorbance for test incubations of 0.11 units (λ = 600 nm). After 24 hours of incubation, absorbance of all test cultures was measured at λ = 600 nm.

5.2.4 Comparison of inhibitory effect of antimony on *C.boidinii* and *C.tropicalis*

Test incubations were prepared as described above for *S.cerevisiae* using malt extract broth as incubation media and potassium antimony tartrate as test substance. Initial absorbances of test incubations were *C.boidinii* 0.18 units and *C.tropicalis* 0.45 units (λ = 600 nm).

5.2.5 Surface binding and uptake of antimony; *S.cerevisiae* and *R.rubra*

Malt extract broth (3 x 400 ml) was inoculated with a turbid cell suspension of *S.cerevisiae* or *R.rubra* and incubated for 5 days at 28°C, 100 rpm. Following incubation, cultures were combined and culture absorbances were adjusted to *S.cerevisiae* 4.0 units and *R.rubra* 3.9 units, equivalent to a concentration of 13 $mg.ml^{-1}$ dry weight cells. Culture biomass was then concentrated ten-fold by centrifugation to a final volume of 120 ml. 10 ml of the concentrated biomass suspension was dispensed to 15 ml screw-capped, plastic centrifuge tubes. Antimony or arsenic stock solution (1 ml) was added such that a final concentration of 10 $mg.l^{-1}$ metal was achieved. To half of the flasks, 1 ml 0.26 $g.ml^{-1}$ sodium azide was added as an inhibitor of active transport, and 1ml water was dispensed to the remaining flasks to achieve equivalent volume. All centrifuge tubes were vortexed for 30 seconds and 1.3 ml aliquots removed for analysis (t = 0 sample). Tubes were then incubated on their sides in a rotary incubator at room temperature and 250 rpm. 1.3 ml aliquots were removed after 10, 15, 20, 30 and 60 minutes. A sample was additionally removed from "no azide" incubations after 18

hours. Upon removal of samples from the test system, biomass and particulate matter were immediately separated from supernatant by microcentrifugation, 13,000 rpm, 5 minutes. The resulting supernatant was stored at –20°C until inductively coupled plasma-atomic emission spectrometry (ICP-AES) analysis was performed as described in section 3.2.1.

5.2.6 Analysis of volatile and involatile antimony and arsenic species

GC-AAS analysis of volatile, hydride generation-GC-AAS analysis of involatile arsenic and antimony species and HPLC-hydride generation-AFS analysis of involatile antimony species were performed as described before in sections 4.2.12 and 4.2.13.

5.3 Results

5.3.1 Production of involatile methylarsenic species by *Candida* sp.

GC-AAS analysis of hydride generated culture supernatants from *C.boidinii* incubations supplied with inorganic arsenic revealed the presence of involatile trimethylarsenic species (Table 5.1). No mono or dimethylated arsenic species were observed at any time. This contrasts with investigations using *C.humicolus*, for which, in addition to trimethylarsenic, low levels of dimethylarsenic species were observed. Transformation of arsenic from the III valency state was higher than that observed when substrate was supplied in the V valency form. Transformation yields were low, being of the order 0.25% and 0.08% from sodium arsenite and sodium arsenate respectively. This is lower than methylation yields observed using *C.humicolus* where a typical transformation yield of 8% sodium arsenite to involatile methyl species was observed. Transformation yield from dimethylated substrate, i.e. dimethylarsinic acid, was higher than that observed from inorganic substrate. 1.2% of the substrate was transformed to involatile trimethylarsenic species after 23-days incubation. No monomethylated arsenic species were observed in culture supernatants when dimethylarsinic acid was used as transformation substrate, indicating that *C.boidinii* does not possess the capability to perform demethylation. In contrast to the relatively high transformation yield observed using dimethylated arsenic substrate, transformation of monomethylarsonic acid to involatile di-, and trimethylated arsenic species was of a similar order to that observed from inorganic arsenic III substrate. Typically, 0.3% of monomethylarsonic acid was transformed to higher methylated species.

It was noted that the total amount of methylated species, i.e. including remaining substrate, observed in culture supernatants of incubations supplied with methylated substrate, was not equivalent to the amount of methylated substrate initially supplied. Analysis of monomethylarsonic acid, dimethylarsinic acid and trimethylarsenic oxide standards by hydride generation-GC-AAS did not reveal significant differences in hydride generation yields between the three methylated arsenic species in that peak area was approximately equivalent for equivalent methylarsenic loadings. These data, may suggest intracellular accumulation of methylated species as was observed with *C.humicolus*, or that additional alternative arsenic species may be present in culture incubations. These species may be inorganic and therefore removed by the alumina column preparative stage, or may be organoarsenic species that cannot be hydride

generated. A further possibility to consider is that of loss through volatilisation. Analysis of culture headspace gases however, did not reveal the presence of volatile arsenic species in incubations of *Candida* sp..

Table 5.1 Hydride generation-GC-AAS detection of involatile methylarsenic species in culture supernatant of *C.boidinii* incubations.

Substrate	Involatile methylarsenic species ($ng.ml^{-1}$)		
	MeAs	Me_2As	Me_3As
No metal	nd	nd	nd
Sodium arsenite $1mg.l^{-1}$	nd	nd	2.5 (0.6)
Sodium arsenate $1mg.l^{-1}$	nd	nd	0.8
Monomethylarsonic acid $5mg.l^{-1}$	1.3 (0.3)	0.8 (0.2)	14.0 (1.2)
Dimethylarsinic acid $5mg.l^{-1}$	nd	220.3 (17.8)	57.9 (4.9)

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 60 $pg.ml^{-1}$).

Hydride generation-GC-AAS analysis of culture supernatants from *C.tropicalis* incubations displayed identical profiles of involatile methylarsenic species as those observed in *C.boidinii* incubations. That is, trimethylated arsenic species were the only involatile methylarsenic species observed when inorganic arsenic, in either valency, was supplied as the transformation substrate; no monomethylarsenic species were observed in incubations supplied with dimethylarsinic acid and both di- and trimethylated arsenic species were detected in culture supernatants from incubations supplied with monomethylarsonic acid (Table 5.2). Transformation yields from all substrates were of the same order as those observed in *C.boidinii* incubations (Table 5.3).

Table 5.2 Hydride generation-GC-AAS detection of involatile methylarsenic species in culture supernatant of *C.tropicalis* incubations.

Substrate	Involatile methylarsenic species ($ng.ml^{-1}$)		
	MeAs	Me_2As	Me_3As
No metal	nd	nd	nd
Sodium arsenite $1mg.l^{-1}$	nd	nd	1.9 (0.5)
Sodium arsenate $1mg.l^{-1}$	nd	nd	1.3 (0.4)
Monomethylarsonic acid $5mg.l^{-1}$	2.1 (0.4)	7.7 (1.7)	28.3 (5.3)
Dimethylarsinic acid $5mg.l^{-1}$	nd	305.0 (13.1)	86.4 (4.5)

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 60 $pg.ml^{-1}$).

Table 5.3 Comparison of transformation yields observed in *C.boidinii* and *C.tropicalis* incubations for the formation of involatile methylated arsenic species from various arsenic substrates.

Substrate	Transformation yield (%)	
	C.boidinii	*C.tropicalis*
Sodium arsenite 1mg.l^{-1}	0.3	0.2
Sodium arsenate 1mg.l^{-1}	0.1	0.1
Monomethylarsonic acid 5mg.l^{-1}	0.3	0.7
Dimethylarsinic acid 5mg.l^{-1}	1.2	1.7

Transformation yield = (sum of involatile methyl species in supernatant (excluding any methylated substrate) ÷ amount of substrate supplied) x 100.

5.3.2 Biotransformation of antimony by Candida sp.

As with arsenic substrate, no volatile antimony species were detected in culture headspace gases of *C.boidinii* or *C.tropicalis* cultures by GC-AAS analysis of biphasic (aerobic / anaerobic) incubations. Analysis of hydride generated culture supernatants by GC-AAS however, did reveal the presence of monomethylantimony species in biphasic incubations of both *C.boidinii* and *C.tropicalis* that were supplied with potassium antimony tartrate as transformation substrate (Table 5.4). Monomethylantimony was the sole involatile methylantimony species detected in *C.tropicalis* incubations, whilst involatile mono-, di- and trimethylated antimony species were found in *C.boidinii* incubations. No methylated antimony species were detected in the culture supernatant of biphasic incubations supplied with potassium hexahydroxyantimonate at any time. These data are of interest since while it cannot be assumed that the ratio of methylantimony species observed under biphasic incubations will mimic that of fully aerobic conditions, it does demonstrate that these two organisms possess the ability to methylate inorganic antimony.

Analysis of the liquid phase of fully aerobic culture incubations by HPLC-hydride generation-AFS revealed the presence of involatile methylantimony species. Trimethylantimony oxide was the sole involatile species identified in culture supernatants. In contrast to the formation of involatile methylarsenic species, for which the two organisms showed comparable transformation yields, *C.boidinii* displayed a higher capability to methylate antimony than *C.tropicalis*.

Table 5.4 GC-AAS analysis of culture supernatant from biphasic (aerobic / anaerobic) ten-fold biomass-concentrated *C.boidinii* and *C.tropicalis* incubations.

Organism + Substrate	Involatile methylantimony species ($ng.ml^{-1}$)		
	MeSb	Me_2Sb	Me_3Sb
C.boidinii + potassium antimony tartrate	4.8 (0.9)	5.9 (0.8)	1.4 (0.6)
C.boidinii + potassium hexahydroxyantimonate	nd	nd	nd
C.tropicalis + potassium antimony tartrate	0.9 (0.3)	nd	nd
C.tropicalis + potassium hexahydroxyantimonate	nd	nd	nd

Metal was supplied at 100 $mg.l^{-1}$ elemental metal to incubations. figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 20 $pg.ml^{-1}$)

A maximum of 28.5 $ng.ml^{-1}$ trimethylantimony oxide was detected in culture supernatants from *C.boidinii* incubations supplied with potassium antimony tartrate, compared to 4.4 $ng.ml^{-1}$ in culture supernatants from *C.tropicalis* incubations (Table 5.5). No methylantimony species were detected in culture incubations of either organism not supplied with metal substrate, nor were methylantimony species identified in non-biomass incubations. These data demonstrate that the trimethylantimony oxide detected in culture incubations arose biogenically.

Table 5.5 HPLC-hydride generation-AFS detection of involatile methylantimony in culture supernatant from *C.boidinii* and *C.tropicalis* incubations.

Sample	Involatile methylantimony ($ng.ml^{-1}$)
No biomass + potassium antimony tartrate	nd
C.boidinii + no metal	nd
C.boidinii + potassium antimony tartrate	28.5 (8.1)
C.tropicalis + no metal	nd
C.tropicalis + potassium antimony tartrate	4.4 (1.9)

Trimethylantimony oxide was the sole involatile methylantimony species identified in incubations. figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 3.1 $ng.ml^{-1}$); antimony was supplied to incubations at 100 $mg.l^{-1}$ elemental metal.

5.3.3 Resistance of *Candida* sp. to arsenic and antimony

C.tropicalis was observed to be more sensitive to potassium antimony tartrate than *C.boidinii* (Figure 5.1). Increasing the load of antimony III upon cultures from no metal to 5000 $mg.l^{-1}$ elemental metal resulted in a decrease in culture absorbance of 0.48 and

0.96 units for *C.boidinii* and *C.tropicalis* respectively. This equates to a 33% and 60% reduction in respective dry weights for the two fungi. The presence of potassium antimony tartrate in culture incubations of *C.boidinii* appeared to have little effect with regard to inhibition of growth until levels of 500 $mg.l^{-1}$ elemental metal were exceeded. In contrast, amending cultures of *C.tropicalis* with 100 $mg.l^{-1}$ antimony III resulted in a 25% decrease in biomass compared to no metal incubations. Further elevation of antimony loading to 500 $mg.l^{-1}$ resulted in a 48% decrease in biomass levels compared to no metal incubations.

5.3.4 Biotransformation of antimony by *G.candidum*

GC-AAS analysis of culture headspace gases from biphasic (aerobic / anaerobic) biomass-concentrated incubations of *G.candidum* supplied with potassium antimony tartrate or potassium hexahydroxyantimonate did not reveal the presence of volatile antimony species. Hydride generation-GC-AAS analysis of alumina-treated supernatants of these culture incubations revealed the presence of mono-, di- and trimethylated involatile antimony species in incubations supplied with antimony in the III valency (Table 5.6). No involatile methylantimony species were detected at any time in incubations supplied with potassium hexahydroxyantimonate. Monomethylantimony was the predominant species detected in incubations supplied with potassium antimony tartrate. This may however, be an anomaly brought about by the application of anaerobic conditions, i.e. after 6 days aerobic incubation cells were placed under respiratory stress, which may influence changes in the processing of antimony and could lead to cell lysis, releasing partially methylated antimony species to the culture media of *G.candidum* incubations.

Table 5.6 GC-AAS analysis of biphasic (aerobic / anaerobic) ten-fold biomass-concentrated *G.candidum* incubations.

Substrate	Volatile antimony species ($ng.g^{-1}$ biomass)				Involatile antimony species ($ng.ml^{-1}$)		
	SbH_3	MeSb	Me_2Sb	Me_3Sb	MeSb	Me_2Sb	Me_3Sb
Potassium antimony tartrate	nd	nd	nd	nd	3.6 (0.8)	0.8 (0.4)	1.2 (0.4)
Potassium hexahydroxyantimonate	nd	nd	nd	nd	nd	nd	nd

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 1 ng absolute). Antimony substrate was supplied to incubations at 50 $mg.l^{-1}$ elemental metal. Involatile antimony species were hydride generated from culture supernatant prior to GC-AAS analysis.

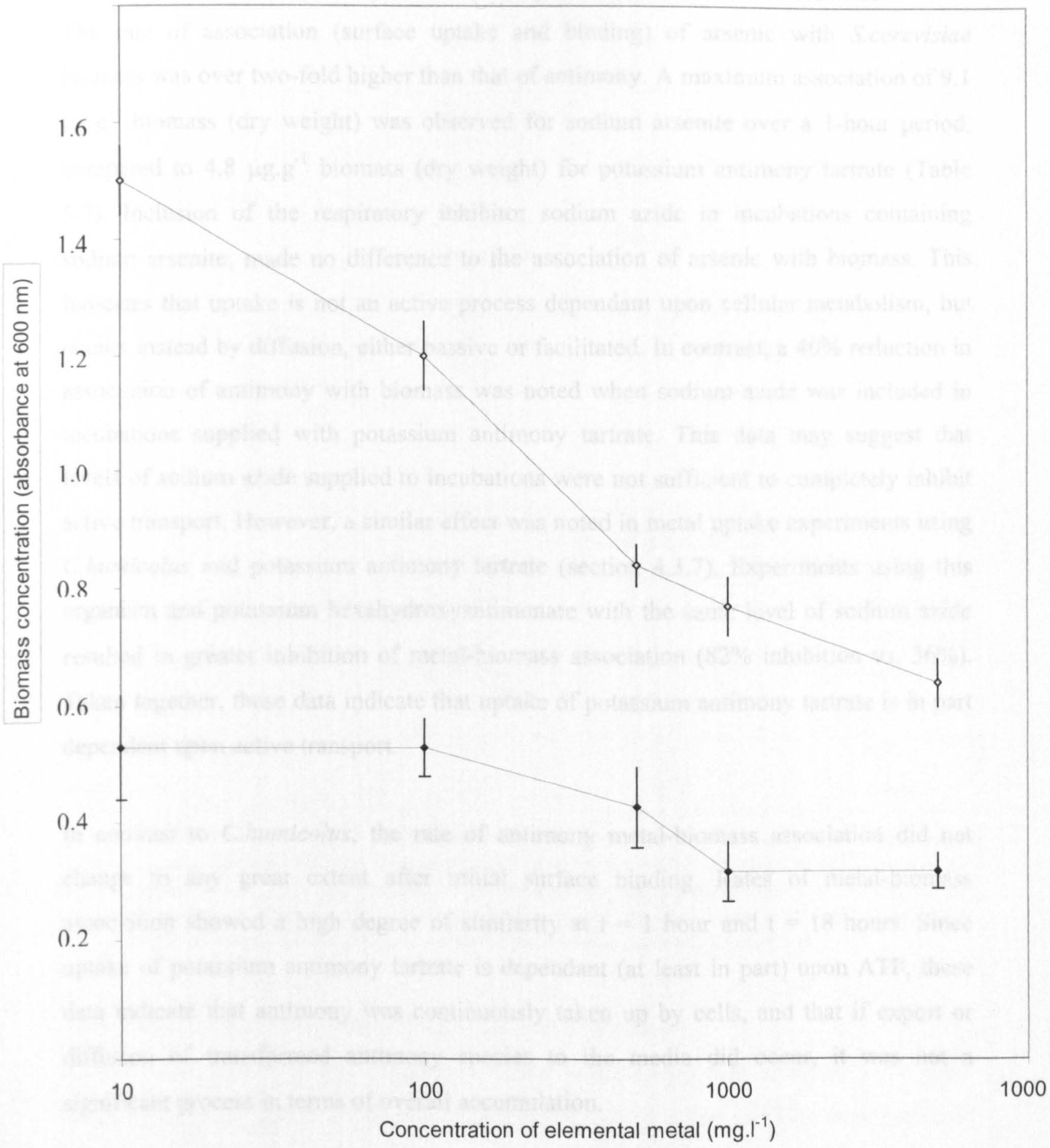


Figure 5.1 Effect of increasing antimony load on growth of (◆) *C.boidinii* and (◇) *C.tropicalis*. Antimony was supplied as potassium antimony tartrate. 10 ml malt extract medium amended with the appropriate concentration of test metal was inoculated with 0.1ml overnight culture inoculum and incubated for 24 hours, after which time absorbance (λ = 600 nm) was recorded as a measure of biomass. A_{600} of no metal reference cultures, *C.boidinii* = 0.8 units, *C.tropicalis* = 1.6 units.

5.3.5 Association of antimony and arsenic species with *S.cerevisiae* biomass

The rate of association (surface uptake and binding) of arsenic with *S.cerevisiae* biomass was over two-fold higher than that of antimony. A maximum association of 9.1 $\mu g.g^{-1}$ biomass (dry weight) was observed for sodium arsenite over a 1-hour period, compared to 4.8 $\mu g.g^{-1}$ biomass (dry weight) for potassium antimony tartrate (Table 5.7). Inclusion of the respiratory inhibitor sodium azide in incubations containing sodium arsenite, made no difference to the association of arsenic with biomass. This indicates that uptake is not an active process dependant upon cellular metabolism, but occurs instead by diffusion, either passive or facilitated. In contrast, a 40% reduction in association of antimony with biomass was noted when sodium azide was included in incubations supplied with potassium antimony tartrate. This data may suggest that levels of sodium azide supplied to incubations were not sufficient to completely inhibit active transport. However, a similar effect was noted in metal uptake experiments using *C.humicolus* and potassium antimony tartrate (section 4.3.7). Experiments using this organism and potassium hexahydroxyantimonate with the same level of sodium azide resulted in greater inhibition of metal-biomass association (82% inhibition vs. 36%). Taken together, these data indicate that uptake of potassium antimony tartrate is in part dependent upon active transport.

In contrast to *C.humicolus*, the rate of antimony metal-biomass association did not change to any great extent after initial surface binding. Rates of metal-biomass association showed a high degree of similarity at t = 1 hour and t = 18 hours. Since uptake of potassium antimony tartrate is dependant (at least in part) upon ATP, these data indicate that antimony was continuously taken up by cells, and that if export or diffusion of transformed antimony species to the media did occur, it was not a significant process in terms of overall accumulation.

Table 5.7 Association of inorganic antimony and arsenic with *S.cerevisiae*.

A

Compound	Initial rate of metal-biomass association t = 0 - 5min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 5 - 60min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 1 - 18h ($\mu g.g^{-1}.min^{-1}$)
Potassium antimony tartrate	0.14	0.05	0.04
Sodium arsenite	0.25	0.12	0.01

B

Compound	Initial rate of metal-biomass association t = 0 - 5min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 5 - 60min ($\mu g.g^{-1}.min^{-1}$)	Reduction in metal-biomass association rate when incubated with sodium azide
Potassium antimony tartrate	0.19	0.03	40%
Sodium arsenite	0.25	0.13	no change

Reaction mixture in malt extract media (volume 10 ml) contained (A) *S.cerevisiae* 1.3 g dry weight biomass; metal substrate 100 μg; and (B) *S.cerevisiae* 1.3 g dry weight biomass; metal substrate 120 μg; 26 μg sodium azide. Final biomass association after 18-hours incubation (no sodium azide); potassium antimony tartrate 44.25 (3.76) $\mu g.g^{-1}$ (dry weight biomass); sodium arsenite 18.05 (1.18). Figure in parentheses represents standard deviation of three replicate incubations.

5.3.6 Resistance of *S.cerevisiae* to arsenic and antimony

As for *C.humicolus*, arsenic supplied in either valency state was observed to be more toxic, in terms of growth inhibition, than antimony. In addition, the III valency state of both metals inhibited growth more than the V valency state (Figure 5.3). Amendment of incubations with antimony III and V at 5000 $mg.l^{-1}$ resulted in a decrease in biomass levels of 39% and 21% respectively, compared to non-amended incubations. This is far lower than the inhibitory effect observed when arsenic was supplied to incubations. Amendment at 10 $mg.l^{-1}$ resulted in a 60% and 44% reduction compared to non-amended incubations for arsenic III and arsenic V respectively, whilst amendment at 5000 $mg.l^{-1}$ of either valency resulted in complete growth inhibition.

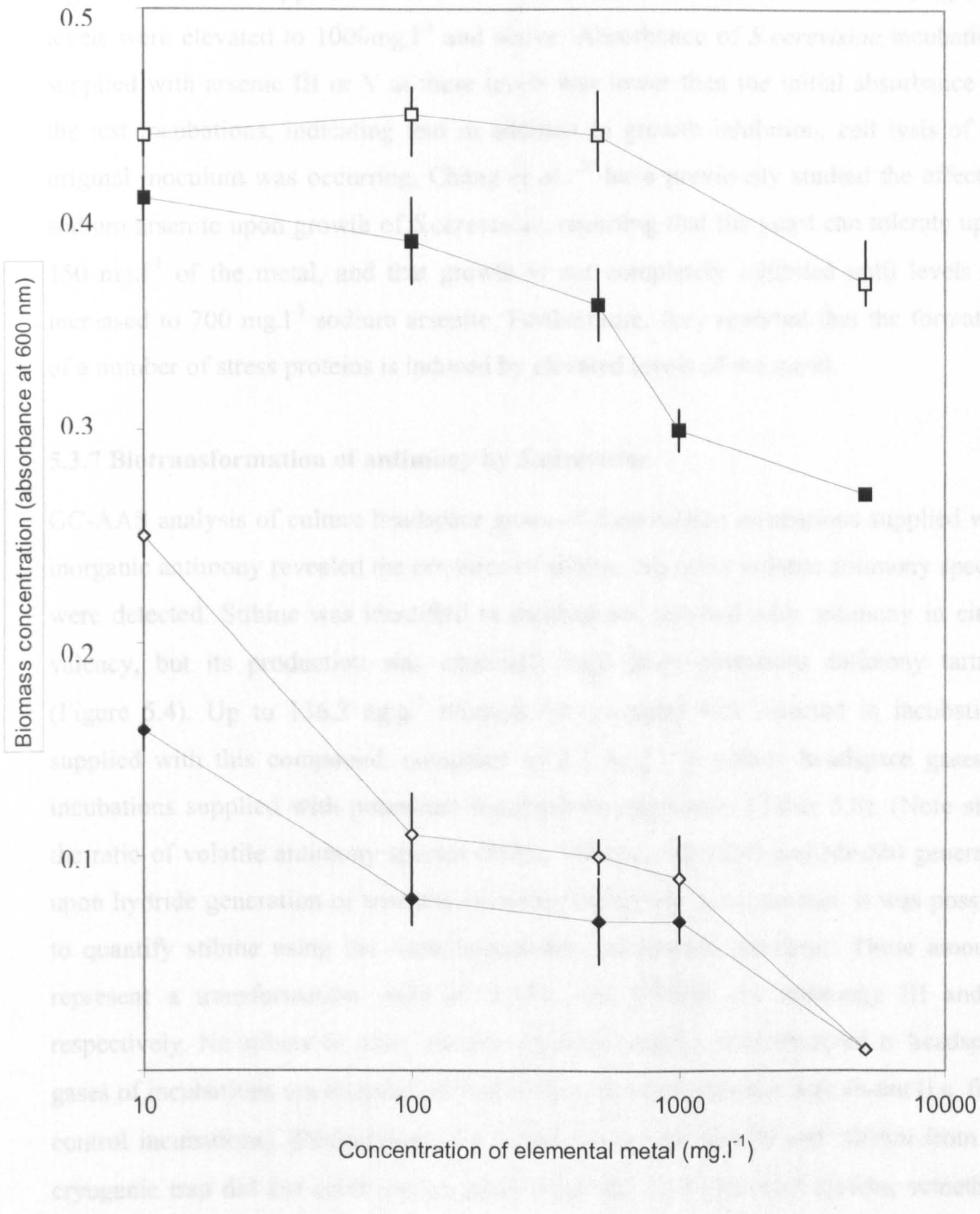


Figure 5.3 Effect of increasing antimony and arsenic load on growth of *S.cerevisiae*. Antimony was supplied as (■) potassium antimony tartrate or (□) potassium hexahydroxyantimonate. Arsenic was supplied as (◆) sodium arsenite or (◇) sodium arsenate. 10 ml malt extract medium amended with the appropriate concentration of test metal was inoculated with 0.1ml overnight culture inoculum and incubated for 24 hours, after which time absorbance (λ = 600 nm) was recorded as a measrure of biomass. A_{600} of no metal reference culture = 0.48 units.

Interestingly, despite a reduction in biomass of 80% when arsenic was supplied at 100 $mg.l^{-1}$, *S.cerevisiae* appeared able to tolerate further increases in arsenic loading until levels were elevated to 1000$mg.l^{-1}$ and above. Absorbance of *S.cerevisiae* incubations supplied with arsenic III or V at these levels was lower than the initial absorbance for the test incubations, indicating that in addition to growth inhibition, cell lysis of the original inoculum was occurring. Chang *et al.* [26] have previously studied the effect of sodium arsenite upon growth of *S.cerevisiae*, reporting that the yeast can tolerate up to 150 $mg.l^{-1}$ of the metal, and that growth is not completely inhibited until levels are increased to 700 $mg.l^{-1}$ sodium arsenite. Furthermore, they reported that the formation of a number of stress proteins is induced by elevated levels of the metal.

5.3.7 Biotransformation of antimony by *S.cerevisiae*

GC-AAS analysis of culture headspace gases of *S.cerevisiae* incubations supplied with inorganic antimony revealed the presence of stibine. No other volatile antimony species were detected. Stibine was identified in incubations supplied with antimony in either valency, but its production was especially high from potassium antimony tartrate (Figure 5.4). Up to 136.3 $ng.g^{-1}$ biomass (dry weight) was detected in incubations supplied with this compound, compared to 3.3 $ng.g^{-1}$ in culture headspace gases of incubations supplied with potassium hexahydroxyantimonate (Table 5.8). (Note since the ratio of volatile antimony species (SbH_3, $MeSbH_2$, Me_2SbH and Me_3Sb) generated upon hydride generation of trimethylantimony dichloride was constant, it was possible to quantify stibine using the trimethylantimony dichloride standard.) These amounts represent a transformation yield of 0.27% and 0.007% for antimony III and V respectively. No stibine or other volatile antimony species were observed in headspace gases of incubations not supplied with antimony, or when biomass was absent (i.e. from control incubations). Furthermore, the release of carbon dioxide and ethanol from the cryogenic trap did not elicit any response from the AAS detection system, something for which there was initial concern.

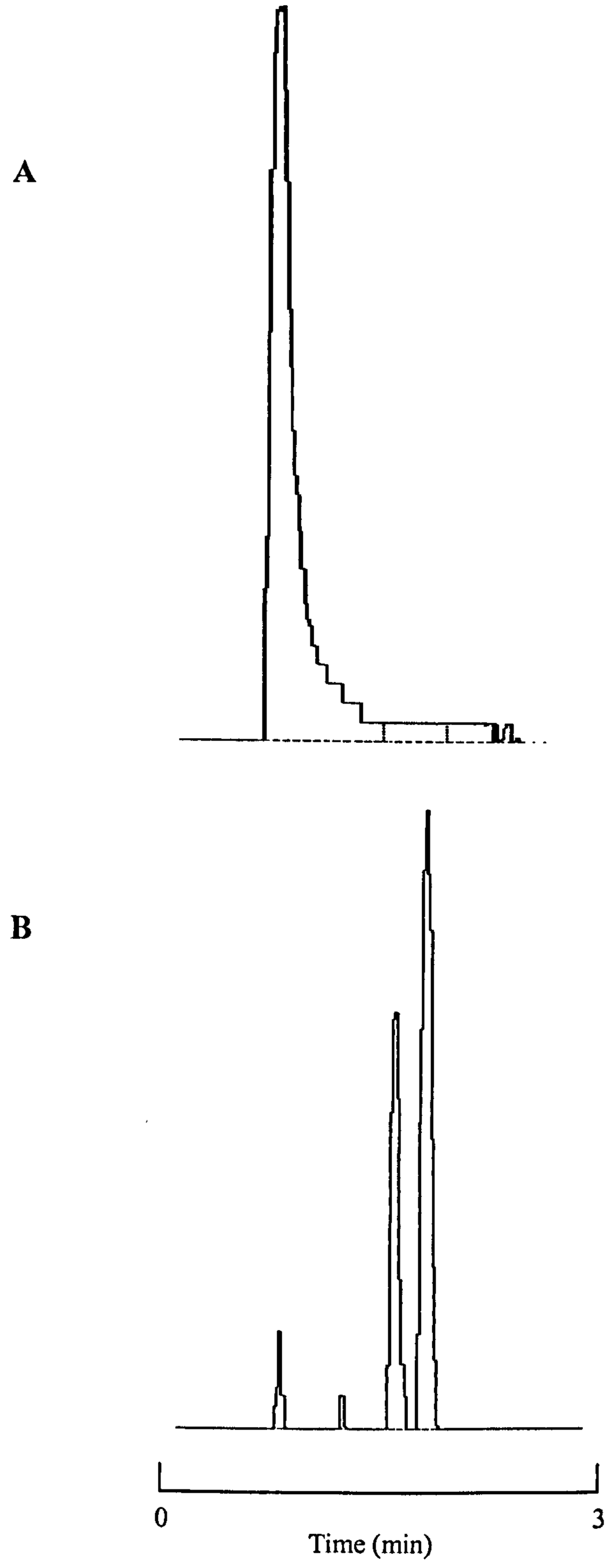


Figure 5.4 GC-AAS identification of stibine (SbH_3) in culture headspace gases of biphasic (aerobic / anaerobic) *S.cerevisiae* incubations (ten-fold biomass-concentrated). (A) Typical spectra of headspace gases of 23-day *S.cerevisiae* incubation. (B) Spectra of antimony standards obtained by distal hydride generation of trimethylantimony dichloride and purging of resultant volatiles to a liquid nitrogen cooled trap. Retention times of standards are; stibine 0.79 min, monomethylstibine 1.33 min, dimethylstibine 1.71 min, trimethylstibine 1.94 min.

Table 5.8 Volatilisation of antimony from inorganic antimony substrate by *S.cerevisiae*. GC-AAS analysis

Substrate	Volatile methylantimony species ($ng.g^{-1}$ biomass)			
	SbH_3	MeSb	Me_2Sb	Me_3Sb
No metal	nd	nd	nd	nd
Potassium antimony tartrate	136.3 (6.8)	nd	nd	nd
Potassium hexahydroxyantimonate	3.3 (1.1)	nd	nd	nd
Potassium antimony tartrate, no biomass	nd	nd	nd	nd

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 1 ng absolute); antimony supplied to incubations at 50 $mg.l^{-1}$ elemental metal.

Involatile mono- and dimethylated antimony species were detected by hydride generation-GC-AAS in biphasic (aerobic / anaerobic) *S.cerevisiae* incubations supplied with antimony. No trimethylated species were detected at any time (Table 5.9). The level of monomethylantimony was higher than amounts of dimethylated antimony species in incubations supplied with antimony in the III valency. When antimony was supplied as potassium hexahydroxyantimonate amounts of the two species were roughly equivalent. These data demonstrate that *S.cerevisiae* possesses a biomethylating capability with regard to antimony.

Table 5.9 GC-AAS detection of involatile methylantimony species in culture supernatant of *S.cerevisiae* incubations biphasic (aerobic / anaerobic) incubations.

Substrate	Involatile methylantimony species ($ng.ml^{-1}$)		
	MeSb	Me_2Sb	Me_3Sb
No metal	nd	nd	nd
Potassium antimony tartrate	1.2 (0.5)	0.3 (0.2)	nd
Potassium hexahydroxyantimonate	0.3 (0.2)	0.4 (0.2)	nd

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 20 $pg.ml^{-1}$); antimony supplied to incubations at 50 $mg.l^{-1}$ elemental metal.

5.3.8 Biotransformation of inorganic arsenic by *S.cerevisiae*

As with incubations supplied with antimony, the volatile reduced metal hydride, i.e. arsine, was the sole volatile arsenic species observed in culture headspace gases of incubations supplied with inorganic arsenic. Levels of arsine were again higher from the III valency substrate, however differences in transformation yields from the two valency

states were not as marked as those observed from antimony substrate. This may be due to differences in uptake rates of the various species. Up to of 16.3 ng.ml^{-1} arsine was detected in culture headspace gases of *S.cerevisiae* incubations supplied with sodium arsenite, compared to 4.9 ng.ml^{-1} in incubations supplied with sodium arsenate (Table 5.10). These equate to transformation yields of 1.63% and 0.49% respectively. No involatile methylated arsenic species were detected at any time in *S.cerevisiae* incubations supplied with inorganic arsenic.

Table 5.10 GC-AAS analysis of culture headspace gases from biphasic (aerobic / anaerobic) *S.cerevisiae* incubations supplied with inorganic arsenic substrate.

Substrate	Volatile methylarsenic species (ng.g^{-1} biomass)			
	AsH_3	MeAs	Me_2As	Me_3As
No metal	nd	nd	nd	nd
Sodium arsenite	16.3 (0.6)	nd	nd	nd
Sodium arsenate	4.9 (1.0)	nd	nd	nd
Sodium arsenite, no biomass	nd	nd	nd	nd

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 3 ng absolute); arsenic supplied to incubations at 1 mg.l^{-1} elemental metal.

5.3.9 Biotransformation of inorganic antimony and arsenic by *R.rubra*

HPLC-hydride generation-AFS analysis of culture supernatants from *R.rubra* incubations supplied with potassium antimony tartrate revealed the presence of an involatile antimony species of retention time 2.4 minutes (Figure 5.5). No involatile antimony species were detected in culture supernatants that were passed through alumina columns, i.e. no methylantimony species were detected in culture supernatants. Furthermore inorganic antimony III did not elute from the column under the conditions used, indicating that the observed antimony species is an inorganic antimony V species. Up to 439.9 ng.ml^{-1} inorganic antimony V was detected in culture supernatants, which equates to a transformation yield of 0.44%. GC-AAS analysis of hydride generated culture supernatants did not reveal the presence of any methylated antimony species, nor were any volatile antimony species, stibine or methylantimony, detected in culture headspace gases. Likewise, hydride generation-GC-AAS analysis did not reveal the presence of involatile methylarsenic species in culture supernatants of incubations supplied with inorganic arsenic as transformation substrate.

5.3.10 Association of arsenic III with *R.rubra* biomass

As was observed with *C.humicolus* and *S.cerevisiae*, uptake of sodium arsenite by *R.rubra* is a metabolism independent process. Inclusion of sodium azide in incubations of *R.rubra* and sodium arsenite resulted in little change in rates of biomass-metal association compared to incubations containing no sodium azide (Table 5.11). In contrast to *S.cerevisiae*, but consistent with investigations using *C.humicolus*, a decrease in the rate of biomass-metal association was noted as the incubation progressed. This indicates that uptake of the species occurs by facilitated diffusion, the continued uptake of sodium arsenite being driven by its subsequent intracellular transformation.

Table 5.11 Association of inorganic antimony and arsenic with *R.rubra*.

Compound	Initial rate of metal-biomass association t = 0 - 5min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 5 - 60min ($\mu g.g^{-1}.min^{-1}$)	Rate of metal-biomass association t = 1 - 18h ($\mu g.g^{-1}.min^{-1}$)
Sodium arsenite	0.14	0.07	0.01
Sodium arsenite + sodium azide	0.12	0.06	na

Reaction mixture in malt extract media (volume 10 ml) contained *R.rubra* 1.3 g dry weight biomass and metal substrate 120 μg. Sodium azide was added to comparison incubations to inhibit active transport at 26 μg; na = not analysed. Final biomass association after 18-hours incubation (no sodium azide 14.75 (3.46) $\mu g.g^{-1}$ (dry weight biomass). Figure in parentheses represents standard deviation of three replicate incubations.

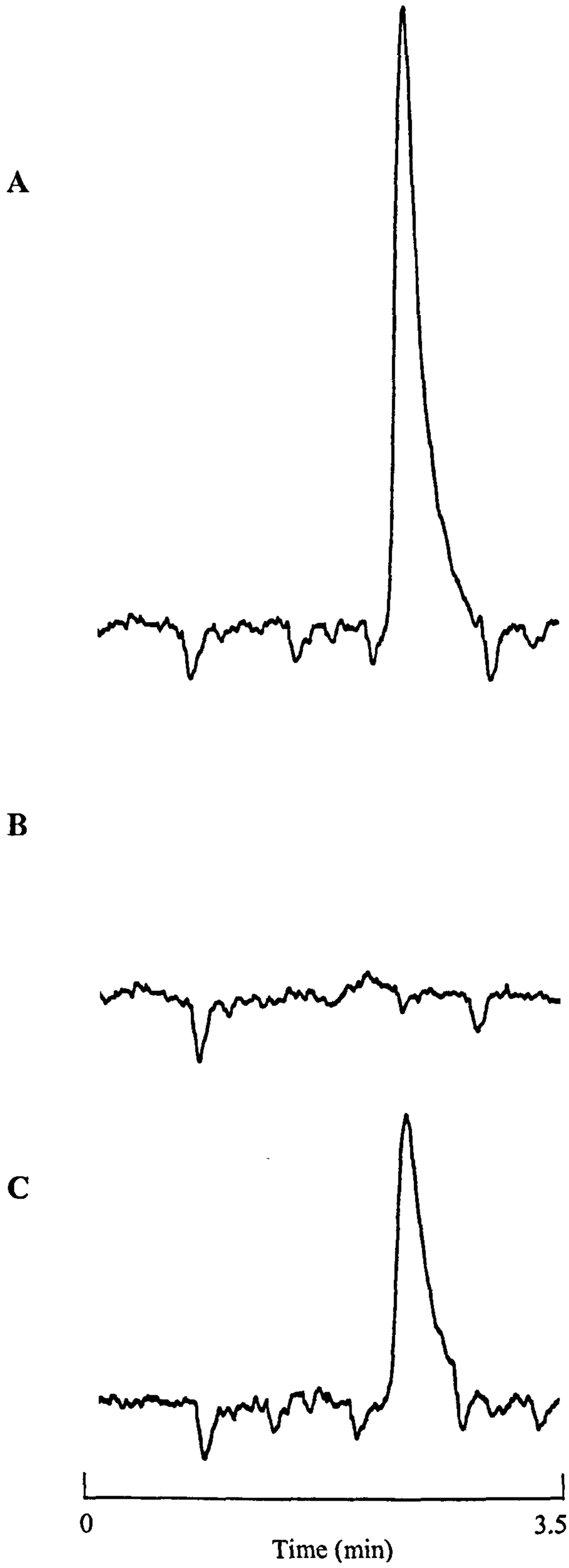


Figure 5.5 HPLC-hydride generation-AFS detection of inorganic antimony V in culture supernatants of *R.rubra* incubations supplied with potassium antimony tartrate as transformation substrate. (A) Typical spectra of culture. (B) Typical spectra of culture supernatant cleaned of inorganic antimony by passage through a basic alumina column. (C) Spectra of antimony V standard potassium hexahydroxyantimonate, 1 ng loading retention time 2.4 minutes.

5.3.11 Volatilisation of antimony and arsenic by *S.brevicaulis*

GC-AAS analysis of culture headspace gases of *S.brevicaulis* incubations supplied with inorganic antimony revealed the presence of volatile antimony species. Trimethylantimony was the sole volatile antimony species detected in cultures supplied with potassium antimony tartrate as transformation substrate. A maximum of 25.7 $ng.g^{-1}$ biomass (dry weight), amounting to a transformation yield of 0.05% was detected (Table 5.12). No stibine or other volatile methylantimony species were detected in such incubations. In contrast, stibine was detected in incubations supplied with antimony V as potassium hexahydroxyantimonate; volatile methylantimony species were not detected. The amount of volatile species present in incubations supplied with antimony V was lower than that observed for incubations supplied with antimony in the 3-valency form. A maximum of 7.2 ng stibine per g biomass (dry weight), amounting to a transformation yield of 0.01%, was detected in potassium hexahydroxyantimonate incubations. No volatile antimony species were detected in control incubations, i.e. in the absence of biomass or metal substrate

Table 5.12 Volatilisation of antimony from inorganic substrate by *S.brevicaulis*; GC-AAS detection of volatile antimony species in culture headspace gases of biphasic (aerobic / anaerobic) incubations.

Substrate	Volatile methylantimony species ($ng.g^{-1}$ biomass)			
	SbH_3	MeSb	Me_2Sb	Me_3Sb
No metal	nd	nd	nd	nd
Potassium antimony tartrate	nd	nd	nd	25.7 (3.0)
Potassium hexahydroxyantimonate	7.2 (1.3)	nd	nd	nd
Potassium antimony tartrate, no biomass	nd	nd	nd	nd
Potassium hexahydroxyantimonate, no biomass	nd	nd	nd	nd

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 1 ng absolute) ; antimony supplied to incubations at 50 $mg.l^{-1}$ elemental metal.

Volatilisation of inorganic arsenic by *S.brevicaulis* showed a similar profile to that seen from antimony compounds, in that formation of the reduced metal hydride, i.e. arsine, was higher from arsenic V substrate and the formation of trimethylarsine was higher from the III valency substrate (Table 5.13). In contrast to volatilisation from inorganic antimony compounds, both arsine and trimethylarsine were detected in culture headspace gases of biphasic incubations supplied with inorganic substrate of either

valency. A maximum of 6.8 ng arsine per g biomass (dry weight) was detected in incubations supplied with sodium arsenite compared to 13.8 $ng.g^{-1}$ biomass from sodium arsenate. Amounts of trimethylarsine detected in incubations were higher than those of arsine, 22.7 $ng.g^{-1}$ biomass and 18.8 $ng.g^{-1}$ biomass were detected in incubations supplied with sodium arsenite and sodium arsenate respectively. These amounts translate to transformation yields of 0.68% and 1.38% for arsine and 2.27% and 1.88% for trimethylarsine from sodium arsenite and sodium arsenate respectively. No volatile metal species were detected in headspace gases of incubations not supplied with metal substrate, or in incubations devoid of biomass. As was noted with volatilisation of antimony and arsenic by *S.cerevisiae*, volatilisation from arsenic substrates was far higher than that from antimony.

Table 5.13 Volatilisation of arsenic from inorganic substrate by *S.brevicaulis*; GC-AAS detection of volatile arsenic species in culture headspace gases of biphasic (aerobic / anaerobic) incubations.

Substrate	Volatile methylarsenic species ($ng.g^{-1}$ biomass)			
	AsH_3	MeAs	Me_2As	Me_3As
No metal	nd	nd	nd	nd
Sodium arsenite	6.8 (1.1)	nd	nd	22.7 (2.5)
Sodium arsenate	13.8 (2.2)	nd	nd	18.8 (3.5)
Sodium arsenite, no biomass	nd	nd	nd	nd
Sodium arsenate, no biomass	nd	nd	nd	nd

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 3 ng absolute); arsenic was supplied to incubations at 1 $mg.l^{-1}$ elemental metal.

5.3.12 Production of involatile methylantimony species by *S.brevicaulis*

Involatile trimethylantimony species were detected by GC-AAS in hydride generated culture supernatants from *S.brevicaulis* incubations supplied with 50 $mg.l^{-1}$ potassium antimony tartrate or 100 $mg.l^{-1}$ potassium hexahydroxyantimonate. The maximum amount of species observed in such incubations was similar, being 11.4 $ng.ml^{-1}$ and 12.2 $ng.ml^{-1}$ in incubations supplied with potassium antimony tartrate and potassium hexahydroxyantimonate respectively (Table 5.14). No other involatile methylantimony species were observed in these incubations. In contrast, when the amount of potassium antimony tartrate was raised to 100 $mg.l^{-1}$, involatile dimethylantimony species were detected in addition to trimethylated. The amount of trimethylantimony species detected in incubations supplied with 100 $mg.l^{-1}$ potassium antimony tartrate was significantly

higher than that observed in incubations supplied with 50 $mg.l^{-1}$ of this compound as transformation substrate; 128.0 $ng.ml^{-1}$ and 11.4 $ng.ml^{-1}$ trimethylantimony respectively.

Table 5.14 GC-AAS detection of involatile methylantimony species in culture supernatant of fully aerobic *S.brevicaulis* incubations.

Substrate	Involatile methylantimony species ($ng.ml^{-1}$)		
	MeSb	Me_2Sb	Me_3Sb
No metal	nd	nd	nd
Potassium antimony tartrate $50mg.l^{-1}$	nd	nd	11.4 (0.4)
Potassium antimony tartrate $100mg.l^{-1}$	nd	23.7 (6.8)	128.0 (10.2)
Potassium hexahydroxyantimonate $100mg.l^{-1}$	nd	nd	12.2 (3.8)

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 20 $pg.ml^{-1}$).

Analysis of culture supernatants from *S.brevicaulis* incubations by HPLC-hydride generation-AFS revealed the presence of involatile methylantimony species. As was observed with GC-AAS analysis of identical incubations, trimethylated antimony (trimethylantimony oxide) was the sole methylated species present in incubations supplied with 50 $mg.l^{-1}$ antimony III substrate (Table 5.15). Analysis of culture supernatants from incubations supplied with $100mg.l^{-1}$ potassium antimony tartrate by HPLC-AFS revealed the presence of an additional compound eluting shortly before trimethylantimony oxide (Figure 5.6). In the absence of standards, and by comparison to GC-AAS spectra of supernatants from identical incubations, this peak is assumed to be a dimethylated antimony species. The total amounts of involatile methylantimony species detected in culture supernatants by hydride generation-GC-AAS and HPLC-hydride generation-AFS were comparable.

Table 5.15 HPLC-hydride generation-AFS detection of involatile methylantimony in culture supernatant of *S.brevicaulis* incubations.

Sample	Involatile methylantimony ($ng.ml^{-1}$)
No metal	nd
Potassium antimony tartrate $50mg.l^{-1}$	9.0 (0.8)
Potassium antimony tartrate $100mg.l^{-1}$	131.2 (24.5)

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 3.1 $ng.ml^{-1}$).

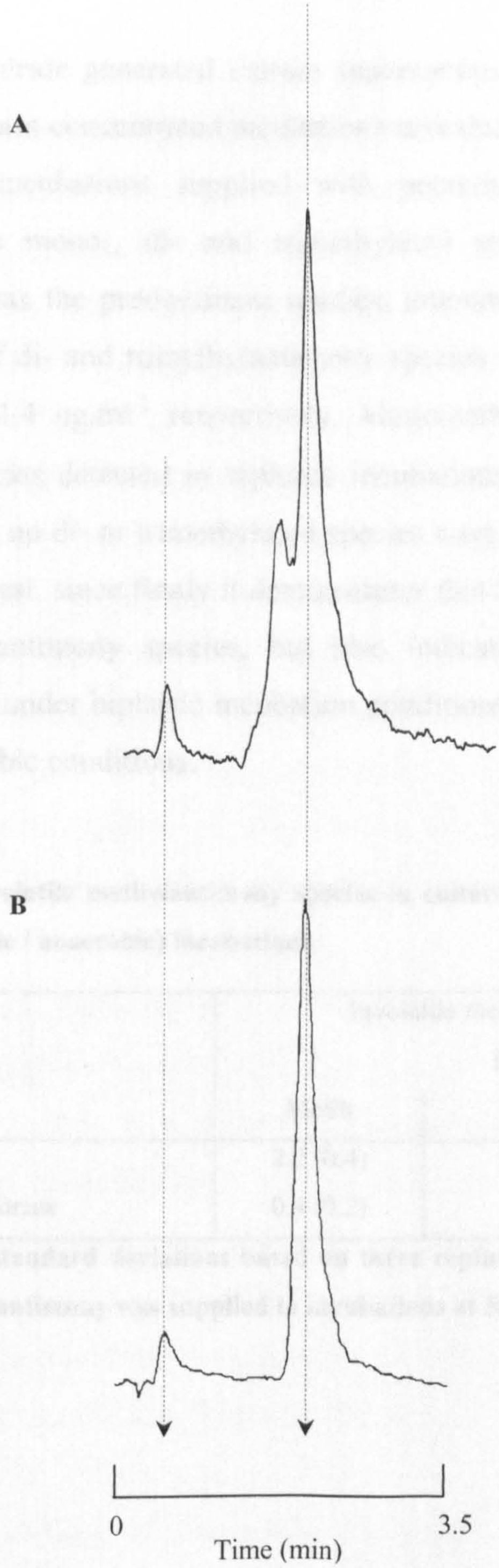


Figure 5.6 Typical chromatograms obtained from HPLC-hydride generation-AFS analysis of culture supernatant from *S.brevicaulis* incubations supplied with potassium antimony tartrate. (A) *S.brevicaulis* + 50ppm potassium antimony tartrate; retention times of peaks: 0.9, 2.2 and 2.4 minutes. (B) Chromatogram of trimethylantimony oxide (1 ng) standard run under identical chromaographic conditions, peak retention times: 0.9 and 2.4 minutes.

GC-AAS analysis of hydride generated culture supernatants from biphasic (aerobic / anaerobic) ten-fold biomass-concentrated incubations revealed the presence of involatile antimony species. In incubations supplied with potassium antimony tartrate as transformation substrate mono-, di- and trimethylated species were all detected. Monomethylantimony was the predominant species, amounting to 2.2 $ng.ml^{-1}$ (Table 5.16), whilst amounts of di- and trimethylantimony species were roughly comparable, being 1.1 $ng.ml^{-1}$ and 1.4 $ng.ml^{-1}$ respectively. Monomethylantimony was the sole involatile antimony species detected in biphasic incubations supplied with potassium hexahydroxyantimonate; no di- or trimethylated species were detected at any time. This data is of particular interest, since firstly it demonstrates that *S.brevicaulis* possesses the ability to form methylantimony species, but also indicates that the formation of methylantimony species under biphasic incubation conditions is different to that taking place under entirely aerobic conditions.

Table 5.16 Detection of involatile methylantimony species in culture supernatant of *S.brevicaulis* incubations biphasic (aerobic / anaerobic) incubations.

Substrate	Involatile methylantimony species ($ng.ml^{-1}$)		
	MeSb	Me_2Sb	Me_3Sb
Potassium antimony tartrate	2.2 (0.4)	1.1 (0.2)	1.4 (0.6)
Potassium hexahydroxyantimonate	0.4 (0.2)	nd	nd

Figure in parentheses are standard deviations based on three replicate culture incubations; nd = not detected (< 20 $pg.ml^{-1}$); antimony was supplied to incubations at 50 $mg.l^{-1}$ elemental metal.

5.4 Discussion

This work has significantly extended the range of pure microorganisms known to methylate antimony. As yet, the only reports of antimony biomethylation by monoseptic cultures regard the fungi *Scopulariopsis brevicaulis* and *Phaeolus schweinitzii*.[6-8, 21, 27] Of the species tested here, only *S.brevicaulis* and *R.rubra* are known to possess a biotransformation capability with regard to arsenic. *S.brevicaulis* is a known arsenic methylator,[13, 17] and *R.rubra* has been shown to reduce arsenate to arsenite and to produce unspecified volatile arsine compound(s).[18] An arsenic (or antimony) biotransformation capability has not previously been described for *Candida* sp. or *Geotrichum* sp, although *C.humicolus*, a member of the same phylogenic order, *Cryptococcaceae*, has previously been demonstrated to methylate arsenic.[16, 17] The antimony methylating capability of this organism has already been described (Chapter 4). *Saccharomyces cerevisiae* has likewise not previously been shown to biotransform either metal. A report of arsenic biomethylation by the closely related yeast *Saccharomyces ellipsoideus* has been made [12] but has been disputed.[13]

The antimony methylating capability of *S.cerevisiae* is particularly interesting, since this was the only ascomycete tested. All other species tested are hyphomycetous anamorphs and produce hyphae to a greater or lesser degree. The establishment of a antimony biomethylating capability for *S.cerevisiae* demonstrates that the ability to biomethylate metals is not linked to hyphae forming or sporulating ability as could be assumed from a perusal of the list of known arsenic methylating fungi (section 4.1). This indicates that, as with bacterial biomethylation of metals, fungal biomethylation is not confined to a specific metabolic or physiological type.

The arsenic methylating abilities of *C.boidinii* and *C.tropicalis* displayed a high degree of similarity. Exposure of cells to inorganic arsenic resulted in the formation of inorganic trimethylarsenic species with the yield of transformation being higher from the III valency form. The detection of involatile dimethylated and trimethylated arsenic species in culture supernatants when monomethylarsonic acid was supplied as substrate, and the detection of only trimethylated arsenic species when dimethylarsinic acid was supplied as substrate, suggests a progression of mono- to di- to trimethylated species as is described by the Challenger mechanism.[13] Both *C.humicolus* and *S.brevicaulis* have been demonstrated to have similar methylation profiles from organoarsenic substrates.[28]

The lack of lower methylated arsenic species when inorganic arsenic was supplied to *Candida* spp. cultures as transformation substrate contrasts with their detection in comparable incubations of *C.humicolus* and *S.brevicaulis*.[28] This may indicate an alternative mechanism of methylation, or more likely occurs as a result of lower substrate transformation yield by the *Candida* spp.. *C.humicolus* typically transformed 8% of sodium arsenite to methylated species compared to around 0.2% for both *C.boidinii* and *C.tropicalis*. It was found through studies with *C.humicolus* that the concentration of involatile dimethylated antimony species in culture supernatant is proportional to the total involatile methylated antimony content of the culture supernatant (section 4.3.12). It is likely that a similar relationship exists with regard to arsenic methylation. Likewise, the non-detection of arsenic volatilisation for *Candida* spp. cultures may not necessarily due to a lack of volatilising capability by these organisms, but may be a factor of the low degree of arsenic biomethylation. For example, Cullen *et al.* [28]noted that the formation of involatile methylarsenic species, and in particular trimethylarsine oxide, far exceeded volatilisation of the metal by cultures of *C.humicolus* and *S.brevicaulis*.

As was noted from the investigations on *C.humicolus*, and has been commented on many times in the literature for the fungus *S.brevicaulis,* antimony biomethylation by *Candida* spp. was lower than of arsenic. Interestingly, the difference in methylation yields from the two metals was not as marked as that observed for *C.humicolus*. Biomethylation by *Candida* spp. from arsenic substrates was ten-fold more efficient, in terms of quantity of product, than from antimony substrate. This compares with a 100-fold difference for *C.humicolus*. These differences in transformation yields of the two metals suggest some degree of genus divergence of the enzymatic systems responsible. Whether this divergence is at the transportation or intracellular level is unclear; although the entry of sodium arsenite into cells is a passive diffusion process entry of potassium antimony tartrate appears to be, at least in part, dependant upon active transport processes.

Species differences in processing of potassium antimony tartrate were observed between the two *Candida* species despite comparable biomass levels in incubations. *C.boidinii* was more efficient than *C.tropicalis* at methylating potassium antimony tartrate to trimethylantimony oxide. It was noted that *C.boidinii* displayed higher resistance to

antimony. It is unlikely however, that this increased resistance is linked to methylation of the metal; methylation yields are so low that this cannot comprise an efficient mechanism for the removal of antimony from the site of cellular metabolism. Furthermore, when antimony resistance profiles obtained for *C.boidinii* and *C.tropicalis* are compared to those of *C.humicolus*, it can be seen that *C.tropicalis* shows a higher degree of similarity to *C.humicolus* than it does to *C.boidinii*. These data do not support the supposition that methylation of antimony is a resistance mechanism in these species.[29]

The identification of a significant capability for stibine (SbH_3) formation from potassium antimony tartrate by *S.cerevisiae* may account for its high degree of resistance to this compound. Volatilisation of large quantities of stibine could serve as an efficient means of antimony removal from cellular biomass. In the absence of metal-biomass association data for antimony in the V valency form, it is impossible to state with any degree of certainty that resistance of *S.cerevisiae* to antimony V is controlled at the uptake level. Control of entry of antimony V to the cell could, however explain the high resistance of the yeast to antimony V since both stibine volatilisation and methylation yields are significantly lower than from antimony III substrate. Certainly, it can be said that antimony volatilisation and biomethylation are not methods of resistance to antimony V substrates employed by *S.cerevisiae*. The absence of volatile methylantimony species in culture headspace gases of *S.cerevisiae* incubations, albeit coupled with the detection of involatile methylantimony species, and comparably high stibine volatilisation, suggests that processing of antimony by *S.cerevisiae* is different to that of *C.humicolus*.

S.cerevisiae volatilised arsine from inorganic substrate as it did from antimony. As is generally noted with biomethylation, volatilisation from arsenic was far higher than from antimony. A six-fold difference was noted between the III valency forms versus a 70-fold difference between the V valency forms of the two metals. This probably reflects differences in the uptake and surface binding of the various species. A comparison of metal-biomass association rates of sodium arsenite and potassium antimony tartrate revealed a two-fold difference with sodium arsenite associating with the cell more rapidly than the antimony compound. The decrease in the rate of sodium arsenite-biomass association with time and lack of inhibitory effect by the respiratory

inhibitor sodium azide, indicates that uptake of sodium arsenite occurs by facilitated rather than passive diffusion. Despite this export, uptake of arsenic III still exceeds export. Little difference in the rate of metal-biomass association for antimony was noted after initial surface binding of the metal to the cell surface was complete. This is in marked contrast to findings with *C.humicolus*. Uptake of both potassium hexahydroxyantimonate and potassium antimony tartrate is dependant (at least in part) upon ATP. Taken together, these data indicate that *S.cerevisiae* does not actively pump antimony species out to the culture media. Since volatilisation levels are lower than uptake of antimony, these data suggest intracellular storage of the metal in some form.

In contrast to previous reports of arsenic volatilisation by *S.brevicaulis*,[13, 17] arsine was identified in addition to the presence of trimethylarsine in culture headspace gases. A biphasic (aerobic / anaerobic) culture regime was employed here, which may account for the preservation of these species in the absence of oxygen. It should also be considered that the formation of arsine might be a factor of the physiological stress exerted upon cells by the application of oxygen starvation conditions. The detection of trimethylstibine as sole volatilised arsenic species from potassium antimony tartrate substrate is in agreement with the findings of Jenkins *et al.*[6] Likewise Andrewes *et al.* [7]reported that trimethylstibine was the sole volatile species produced by incubations supplied with potassium antimony tartrate alone. As yet, no speciation of antimony volatiles produced by *S.brevicaulis* in incubations supplied with antimony V substrate alone has been reported in the literature.

S.brevicaulis was shown here to produce involatile methylantimony species from inorganic antimony substrate if supplied in either the III or V valency forms. Both trimethylantimony oxide and an unknown dimethylated antimony compound were detected. This, and the detection of biogenic stibine in headspace gases of *S.brevicaulis* incubations amended with potassium hexahydroxyantimonate, counteract the claim of Andrewes *et al.* [7] that fungi are unable to transport or methylate antimony V substrates. It should be noted that within the same time frame as the Andrewes report, Jenkins *et al* [6] demonstrated biogenic mobilisation of antimony by *S.brevicaulis* from antimony V substrate (antimony pentoxide) to distal HNO_3 traps. Speciation of compounds mobilised from antimony V substrate was not however presented.

Methylation of antimony by *S.brevicaulis* was more favourable from antimony supplied in the III valency form. Jenkins *et al.* [6] suggested this might reflect a requirement for pre-reduction of antimony V to antimony III prior to methylation. The observation that stibine or arsine production was higher when antimony or arsenic was supplied in the V valency state suggests that this organism possess the ability to oxidise antimony III to antimony V. The possibility of bio-oxidation by this organism has been mentioned previously, although supporting data was not presented.[7] This contrasts with the observation of higher levels of formation of stibine or arsine from substrate in the III valency form by *S.cerevisiae*, which could indicate that differences in the intracellular processing of antimony in these two species occurs.

No involatile monomethylantimony species were detected at any time in fully aerobic *S.brevicaulis* incubations. This and the differences in ratios of di- and trimethylated antimony species observed in culture supernatants of *S.brevicaulis* and for example, *C.humicolus* incubations, and fully aerobic and biphasic incubations of *S.brevicaulis* indicates that the involatile monomethylantimony and dimethylantimony species detected in culture incubations do not arise through oxidation of volatile trimethylstibine as has been previously suggested.[30] Similar observations have been previously reported.[7, 8]

The bio-oxidation of antimony by *R.rubra* is of particular interest since this comprises the first report of fungal bio-oxidation of this metal. This may comprise an alternative resistance mechanism to antimony. Microbial resistance to arsenic by oxidation of the metal is well known.[31, 32] To date, the only reports of antimony bio-oxidation are those of Lyalikova [33]who described the growth of *Stibiobacter senarmontii* by deriving energy from the oxidation of antimony trioxide, and Torma and Gabra who detailed the bio-oxidation of antimony by and adapted strain of *Thiobacillus ferrooxidans*.[34] *R.rubra* did not appear to possess the ability to methylate or volatilise arsenic or antimony.

In conclusion, the modes of biotransformation of antimony and arsenic varied not only at the macro-taxonomic level, but also at the species level, demonstrating that the ability to biotransform these elements is widespread in the Fungal kingdom, as it is in the Prokaryotae.

References for Chapter 5

1. A. V. Hirner, U. M. Grueter, and J. Kresimon, Metal(oid)organic compounds in contaminated soil. *Fresenius Journal of Analytical Chemistry* **368**, 263 (2000).
2. H. Gürleyük, V. Van Fleet-Stalder, and T. G. Chasteen, Confirmation of the biomethylation of antimony compounds. *Applied Organometallic Chemistry* **11**, 471 (1997).
3. P. N. Gates, H. A. Harrop, J. B. Pridham, and B. Smethurst, Can microorganisms convert methylated antimony trioxide or potassium antimonyl tartrate to methylated stibines? *Science of the Total Environment* **205**, 215 (1997).
4. R. O. Jenkins, P. J. Craig, D. P. Miller, L. C. A. M. Stoop, N. Ostah, and T.-A. Morris, Antimony biomethylation by mixed cultures of micro-organisms under anaerobic conditions. *Applied Organometallic Chemistry* **12**, 449 (1998).
5. K. Michalke, E. B. Wickenheiser, M. Mehring, A. V. Hirner, and R. Hensel, Production of volatile derivatives of metal(loid)s by microflora involved in anaerobic digestion of sewage sludge. *Applied and Environmental Microbiology* **66**, 2791 (2000).
6. R. O. Jenkins, P. J. Craig, W. Goessler, D. Miller, N. Ostah, and K. J. Irgolic, Biomethylation of inorganic antimony compounds by an aerobic fungus: *Scopulariopsis brevicaulis*. *Environmental Science and Technology* **32**, 882 (1998).
7. P. Andrewes, W. R. Cullen, J. Feldmann, I. Koch, P. E., and K. J. Reimer, The production of methylated organoantimony compounds by *Scopulariopsis brevicaulis*. *Applied Organometallic Chemistry* **12**, 827 (1998).
8. P. Andrewes, W. R. Cullen, E. Polishchuk, and K. J. Reimer, Antimony biomethylation by the wood rotting fungus *Phaeolus schweinitzii*. *Applied Organometallic Chemistry* **15**, 473 (2001).
9. D. K. Barrett, An improved selective medium for isolation of *Phaeolus schweinitzii*. *Transactions of the British Mycological Society* **71**, 507 (1978).
10. M. Slekovec, W. Goessler, and K. J. Irgolic, Inorganic and organic arsenic compounds in Slovenian mushrooms: comparison of arsenic-specific detectors for liquid chromatography. *Chemical Speciation and Bioavailability* **11**, 115 (1999).
11. A. R. Byrne, M. Tusekznidaric, and K. J. Irgolic, Studies of the uptake and binding of trace-metals in fungi 2. Arsenic compounds in *Laccaria amethystina*. *Applied Organometallic Chemistry* **5**, 25 (1991).
12. Verona and *in* F. Challenger, Biological Methylation. *Chemical Reviews* **36**, 315 (1945).
13. F. Challenger, Biological methylation. *Chemical Reviews* **36**, 315 (1945).
14. R. O. Jenkins, P. J. Craig, W. Goessler, and K. J. Irgolic, Biovolatilization of antimony and sudden infant death syndrome (SIDS). *Human & Experimental Toxicology* **17**, 231 (1998).
15. C. R. Paula, A. Purchio, W. Gambale, and B. Correa, Aspectos Biologios das fases filamentosa e leveduriforme de *Scopulariopsis brevicaulis*. *Review of Microbiology* **20**, 483 (1989).
16. D. P. Cox and M. Alexander, Production of trimethylarsine gas from various arsenic compounds by three sewage fungi. *Bulletin of Environmental Contamination & Toxicology* **9**, 84 (1973).

17. W. R. Cullen, C. L. Froese, A. Lui, M. B. C., D. J. Patmore, and M. Reimer, The aerobic methylation of arsenic by microorganisms in the presence of L-methionine-*methyl-d_3*. *Applied Organometallic Chemistry* **139**, 62 (1977).

18. F. V. Vidal and V. M. V. Vidal, Arsenic metabolism in marine bacteria and yeast. *Marine Biology* **60**, 1 (1980).

19. Thom, and Raper, *in* F. Challenger, Biological Methylation. *Chemical Reviews* **36**, 315 (1945).

20. M. L. Bird, F. Challenger, P. T. Charlton, S. J. O., *in* W. R. Cullen, and K. J. Reimer, Arsenic speciation in the environment. *Chemical Reviews* **89**, 713 (1989).

21. P. Andrewes, W. R. Cullen, J. Feldmann, I. Koch, and E. Polishchuk, Methylantimony compound formation in the medium of *Scopulariopsis brevicaulis* cultures: $^{13}CD_3$-L-methionine as a source of the methyl group. *Applied Organometallic Chemistry* **13**, 681 (1999).

22. S. Yannai, L. Duek, D. Merzbach, and I. Berdicevsky, Transformations of mercury by *Saccharomyces cerevisiae*. *in* The Biological Alkylation of Heavy Elements P. J. Craig and F. Glockling, ed., Vol.142. Royal Society of Chemistry, London, (1987).

23. J. S. Thayer, "Environmental chemistry of the heavy elements: hydrido and organo compounds," p. VCH Publishers Inc., Weiheim, Germany, 1995.

24. P. J. Craig and F. Glockling, The biological alkylation of heavy elements. *in* The biological alkylation of heavy elements P. J. Craig and F. Glockling, ed., Vol.1, p. pp 298. Royal Society of Chemistry, London, (1987).

25. S. C. Choi, T. Chase, and R. Bartha, Enzymatic catalysis of mercury methylation by *Desulfovibrio desulfuricans* LS. *Applied and Environmental Microbiology* **60**, 1342 (1994).

26. E. C. Chang, D. J. Kosman, and G. R. Willsky, Arsenic oxide-induced thermotolerance in *Saccharomyces cerevisiae*. *Journal of Bacteriology* **171**, 6349 (1989).

27. R. B. Pearce, M. E. Callow, and L. E. Macaskie, Fungal volatilisation of arsenic and antimony and the sudden infant death syndrome. *FEMS Microbiology Letters* **158**, 261 (1998).

28. W. R. Cullen, H. Li, G. Hewitt, and K. J. Reimer, Identification of extracellular arsenical metabolites in the growth medium of the microorganisms *Apiotrichum humicola* and *Scopulariopsis brevicaulis*. *Applied Organometallic Chemistry* **8**, 303 (1994).

29. O. S. Fatoki, Biomethylation in the natural environment: A review. *South African Journal of Science* **93**, 366 (1997).

30. G. E. Parris and F. E. Brinckman, Reactions which relate to environmental mobility of arsenic and antimony. II. Oxidation of trimethylarsine and trimethylstibine. *Environmental Science & Technology* **10**, 1128 (1976).

31. F. H. Osborne and H. L. Ehrlich, Oxidation of arsenite by soil isolate of *Alcaligenes*. *Journal of Applied Bacteriology* **41**, 295 (1976).

32. M. Shariatpanahi and A. C. Anderson, Effect of sodium arsenate on microbial growth in a chemostat. *Journal of Environmental Science and Health* **16**, 283 (1981).

33. N. N. Lyalikova, Oxidation of trivalent antimony up to the highest oxides as a source of energy for the development of the new autotrophic organism Stibiobacter Gen. N. *Mikrobiologika* **205**, 1228 (1972).

34. A. E. Torma, and G. G. Garbra, *in* A. O. Summers, Microbial transformations of metals. *Annual Review of Microbiology* **32**, 637 (1978).

6 BIOREDUCTION OF ANTIMONY BY ANAEROBIC BACTERIA OF ENVIRONMENTAL ORIGIN AND BY THE KNOWN ARSENATE-RESPIRING BACTERIUM *DESULFOTOMACULUM AURIPIGMENTUM*

6.1 Introduction

Microbial bioreduction of metals is known to be widespread within the natural environment, taking various forms. A number of resistance mechanisms employed by bacteria utilise reductive enzymes, often coupled with active efflux transporters. Arsenate reductase of *Escherichia coli* and *Staphylococcus aureus* reduces arsenate to arsenite, which is subsequently exported by a highly specific membrane-bound anion-transporting ATPase.[1, 2] Resistance to mercury can also arise through bioreduction; some strains of *Pseudomonas, Klebsiella* and *Enterobacter* can enzymatically reduce inorganic mercury Hg^{2+} using mercuric(II) reductase to metallic mercury vapour.[3, 4]

Assimilatory reduction is the reduction of a species with its subsequent incorporation into a biologically mediated macromolcule. In contrast to dissimilatory reduction, it is not an energy generating process. A further difference between dissimilatory and assimilatory reduction is that the dissimilatory reduction process does not incorporate the reduced species into organic material. The most well known assimilatory reduction process is that of nitrogen fixation which is a feature of a number of bacterial species, some of which are symbiotically associated with plants. Assimilatory reduction of arsenic to arsenolipids and arsenosugars has previously been described for several phytoplankton and basidomycetous fungi,[5, 6].

Bioreduction of a number of metals, often in conjunction with biosorption of the resultant reduced species, has been exploited for bioremediation purposes, such as metal leaching and recovery from ore bodies and clean-up of waste effluent from metal processing plants prior to entry to the municipal treatment plant, or dispersion to natural groundwater systems.[7, 8] Linkage of the energy derived from dissimilatory reduction of some metals to the oxidation of a carbon source, i.e. respiration of the metal, has been described for several metals. The utilisation of iron(III), manganese(IV), uranium(VI), selenium(VI) and arsenic(V) as terminal electron acceptor in anaerobic respiration have all been described.[9-12] The range of microbial species demonstrated to possess this faculty is as varied as the number of metals utilised; proteobacteria, vibrio, sulfogens and methanogens have all been demonstrated to utilise novel metallic species as

terminal electron acceptors.[13-16] This demonstrates that the phenomenon is widespread in the microbial world.

Reports of the bioreduction (excluding reductive biomethylation) of antimony are extremely scarce. Gürleyük *et al.* [17] reported the bioreduction of the organoantimony compound trimethyldibromoantimony to trimethylstibine by monoseptic cultures of *Pseudomonas fluorescens*; Michalke *et al.* [18] described the detection of stibine in headspace gases of incubations of the methanogen *Methanobacterium formicium*. There are however, no reports of bacteria or fungi able to derive energy and grow by reduction of antimony.

Considering the toxicological and thermodynamic properties of antimonate, there appears little reason to suppose that antimonate-respiration does not occur within the natural environment. To evaluate the potential for antimony bioreduction within the natural environment, monoseptic strains of anaerobic bacteria were enriched for antimony V respiration capability from a variety of microbial habitats. The capability of a known arsenate-respiring bacterium, *Desulfotomaculum auripigmentum*, to respire or bioreduce antimony was also tested.

6.2 Methodology

6.2.1 Enrichment procedure

Soil samples were collected from a former urban industrial site (Leicester) (not connected with the use of heavy metals) and from a rural field (Lincolnshire). Pond sediment was taken from a public City park (Leicester). All samples were collected from a depth of 5-10 cm below the surface using a corer, with the exception of pond sediment, which was directly sampled with the corer. Samples were stored in sterile screw capped glass bottles. The glass bottles were filled to the top to exclude O_2 and were stored in the dark at room temperature until enrichment cultures were prepared. Total levels of antimony, arsenic, cadmium, chromium, lead, mercury and tin in the soil samples were determined using inductively coupled plasma mass spectrometry (ICP-MS) (Walter Gössler, Karl Franzens Universität, Graz) as described in section 3.2.1.

Bacterial isolates capable of antimony respiration or bioreduction of antimony V as an electron acceptor were enriched by successive sub-cultures in minimal medium containing high levels of antimony V (1220 $mg.l^{-1}$). Antimony minimal medium (Appendix II) was prepared using the Hungate technique, i.e. medium was purged with oxygen-free N_2 and capped with butyl rubber seals and metal crimp caps prior to autoclaving. Reducing agents, for example cysteine hydrochloride, were not added to the medium to ensure that chemical reduction of antimonate did not occur. Soil inoculum was prepared using the method of Honschopp *et al.* [19] i.e. 10 g soil in 75 ml sterile demineralised water, 30 min at 30°C, 250 rpm. Soil inoculum (2 ml) was inoculated to each 100 ml reaction flask containing 95 ml media. Inoculations were made *via* needle and syringe through the septa. All reaction flasks were incubated statically in the dark at 28°C. Enrichments were sub-cultured at four-week periods by transferring 2 ml of gently suspended culture broth to fresh medium.

After four sub-cultures in liquid medium, a number of bacteria were isolated by plating out with successive colony transfers onto antimony minimal medium plates i.e. antimony minimal medium as described above solidified with 2% agar. Agar plates were incubated in an anaerobic jar at 28°C using a CO_2 enriched gas generating kit (Oxoid). The isolates from a single soil sample were distinguishable by size and or colour. Some similarity in colony characteristics was observed between isolates from different soil samples.

6.2.2 Testing of soil isolates for antimony V to antimony III reducing capability

Testing of soil isolates was carried out initially using a crude visually assessed colour assay. Once growth of fresh subcultures prepared from 4-week incubations of soil isolates was established, the agar plates of the 4-week incubation were flooded with phosphomolybdic acid reagent. Agar plates that displayed a blue colouration indicated the presence of antimony III. Subcultures from plates that reacted positively were retained for liquid analysis. All such bacterial isolates were observed to be Gram-positive cocci.

For the second phase of testing, soil isolates were incubated in liquid antimony minimal medium, as described above, for 6-weeks after which time culture supernatants were analysed for the presence of antimony III. The assay used was based on that described by Silicia *et al.*.[20] Kinetic measurements were made using a Perkin Elmer dual beam 555 UV-vis spectrophotometer (beam slit 1nm) fitted with two 1cm path length flow cells. A wavelength scan on the blue heteropoly complex showed that the optimum wavelength for analysis was 750 nm.

The flow cells were supplied continuously (flow rate 30 $ml.min^{-1}$) from external reservoirs that were constantly agitated by magnetic stirring. The minimum operating volume for the system was 10 ml; 2.7 ml solution A (see below), 200 µl phosphomolybdic acid reagent 7.0 ml demineralised water and 100µl of test solution. The reaction was monitored at 750 nm by recording absorbance as a function of time. The test cell was blanked simultaneously *in situ* against a reference cell that contained an equivalent volume of fresh media to the test solution.

Solution A was prepared such that 10 ml contained 1 ml of 5%w/v ascorbic acid, 0.5 ml 10%v/v glycerine and 0.6 ml 0.05% v/v TritonX-100, made up to final volume with demineralised water. Solution A was prepared fresh daily to avoid oxidation of the ascorbic acid. Phosphomolybdic acid reagent (Sigma-Aldrich) was diluted to a working concentration of 5% stock in ethanol.

6.2.3 Utilisation of a range of terminal electron acceptors

All soil isolates were screened with regard to capability to utilise a range of respiratory electron acceptors. Antimony-VT mineral medium (Appendix II) was prepared using a modification of the method of Newmann *et al.*[21] The reducing agent $PdCl_2$ was omitted

to avoid chemical reduction of electron acceptors. Lactate was used as carbon and electron source throughout since it is a non-fermentable carbon substrate. All electron acceptors (excluding oxygen) were supplied to cultures at a concentration of 5 mmol.l^{-1}.

Prior to autoclaving, media was purged with H_2 for a minimum of 30 minutes per litre. Aliquots (30 ml) of medium was dispensed to 35ml reaction vials and sealed with PTFE-lined butyl rubber seals and aluminium crimp caps. Turbid cell inoculum (0.1 ml) was injected through the rubber seal of each flask. All flasks were incubated in the dark at 28°C for 4 weeks, after which time absorbance was measured (λ =600 nm) as an estimate of biomass. Absorbances were background corrected against the average absorbance of three identical incubations omitting biomass.

The following electron acceptors were tested;

Electron acceptor		Supplied as
Se(VI)	SeO_4^{2-}	sodium selenate
Mn(IV)	MnO_2	manganese oxide
As(V)	AsO_4^{3-}	sodium arsenate
Nitrate	NO_3^-	potassium nitrate
Sb(V)	$[Sb(OH)_6]^-$	potassium hexahydroxyantimonate
Sulfate	SO_4^{2-}	magnesium sulfate
Oxygen	O_2	air

6.2.4 Assay for presence of nitrate reductase in soil isolates

Overnight cultures of all soil isolates testing positive for the formation of antimony III were tested to identify whether those isolates were able to utilise nitrate as a terminal electron acceptor, terminate the reaction at nitrite, or able to reduce the nitrate through to ammonium.

Overnight cultures of soil isolates were prepared by inoculating fresh medium as described for the utilisation of a range of electron acceptors with 0.1 ml turbid cell inoculum. Reaction vials were incubated statically in the dark at 28°C for 18 hours, after which time 1 ml of culture supernatant was removed for analysis.

To the 1ml culture supernatant, 1 ml 0.8% sulphanilic acid in 1 mol.l^{-1} acetic acid and 1ml 0.6% dimethyl-a-napthylamine in 1 mol.l^{-1} acetic acid were added. Any culture supernatant that did not display a red colouration, indicating the presence of nitrite, was tested for the further reduction of nitrite to ammonium by the addition of zinc dust. Any nitrate remaining in the media (i.e. has not been converted via nitrite to ammonium) will be reduced by the zinc dust to nitrite, causing a red colouration of the media). Culture supernatants remaining colourless after the addition of zinc dust indicated complete absence of nitrate and therefore reduction to ammonium.

6.2.5 Assay of carbon source and antimonate utilisation and protein formation by soil isolate UW-A

Soil isolate UW-A was examined with regard to the time profile of antimony III and protein formation, and the utilisation of carbon substrate. Antimony-VT mineral medium was prepared as described above for the utilisation of a range of terminal electron acceptors. In addition, a control, no biomass incubation was prepared in an identical manner. All incubations were incubated statically in the dark at 28°C. Samples were removed periodically (every 2 – 3 days) for analysis of (i) lactate, (ii) protein content and (iii) antimony V/ total antimony concentrations.

Analysis of antimony V concentration in culture supernatant

Inorganic antimony V content of culture supernatants was determined by HPLC-hydride generation-AFS analysis using the method of Lintschinger *et al.*[22] This method is detailed in section 4.2.17. Retention time of inorganic antimony V = 2.4 minutes (as determined by retention time of potassium hexahydroxyantimonate standard).

Hydride generation-GC-AAS analysis of culture supernatants.

To establish whether the formation of methylantimony species could account for the reduction in antimony V levels observed throughout the incubation period, hydride generated culture supernatant from day 19 of the incubation was analysed by GC-AAS as described previously (section 3.2.6).

Lactate assay

Lactate was determined in culture supernatants using a lactate assay kit (Sigma-Aldrich). A calibration curve was prepared by diluting 1ml of lactate standard solution

to 10 ml with water. The contents of three NAD vials were each dissolved in 2ml glycine buffer and combined in one vial. To this vial, 0.7 ml water and 0.3 ml Lactate dehydrogenase were mixed. The following dilutions were prepared;

NAD/Glycine/Lactate Dehydrogenase mixture (ml)	Water (ml)	Diluted Lactate Standard (ml)	[Lactate] ($mg.ml^{-1}$)
1.0	2.0	0.0	0
1.0	1.9	0.1	0.12
1.0	1.7	0.3	0.36
1.0	1.5	0.5	0.60
1.0	1.2	0.8	0.96
1.0	1.0	1.0	1.20

All tubes were incubated at 37°C for 15 minutes. After which time absorbance of the lactate standards was read at $\lambda = 340$ nm using lactate = 0 $mg.ml^{-1}$ as reference blank, on a UV-vis spectrophotmoter UV2 (ATI Unicam, Cambridge, Cambs, UK). The calibration graph obtained was a straight line passing through the origin, having the equation $y = 0.6429x$, and $R^2 = 0.999$.

Samples were prepared for analysis by diluting 1 in 10 and dispensing 2ml of diluted sample to a centrifuge tube containing 4 ml 10% trichloroacetic acid (TCA). After vortex mixing for 30 seconds, samples were maintained at 5°C for 5 minutes to ensure complete protein precipitation. Samples were then centrifuged at 13000 rpm and supernatant removed for lactate analysis.

The appropriate number of NAD vials was reconstituted with 2 ml glycine, 4 ml water and 0.1 ml lactate dehydrogenase, and the resultant solutions combined to one vial. 2.9 ml of this solution was pipetted into test and blank cuvettes, and 0.1 ml 10% TCA was added to the blank cuvette. Deproteinised culture supernatant (0.1 ml) was dispensed to the test cuvettes. All cuvettes were mixed by inversion and incubated at 37°C for 15 minutes. After which time absorbance was measured as for standards. Lactate concentration of supernatants was calculated with respect to the calibration graph.

Protein assay

A standard protein curve was prepared by diluting 0.4 $mg.ml^{-1}$ bovine serum albumin (BSA) 1 in 2 with demineralised water to a final concentration of 0.2 $mg.ml^{-1}$ and a calibration curve prepared as follows;

Diluted BSA standard (ml)	Water (ml)	[BSA] ($\mu g.ml^{-1}$)
0	0.5	0
0.1	0.4	40
0.2	0.3	80
0.3	0.2	120
0.4	0.1	160
0.5	0	200

The calibration graph obtained was a straight line passing through the origin, having the equation $y = 0.0026x$, and $R^2 = 0.992$.

Culture broth was concentrated ten-fold by centrifugation, in that 500 µl of culture broth was dispensed to a microcentrifuge and microcentrifuged at 13000 rpm for 10 minutes. Supernatant (450 µl) was discarded and the remaining pellet and supernatant resuspended. Aliquots (6.5 µl) of 20% NaOH was added to the 50 µl of ten-fold concentrated culture broth in the microcentrifuge tube and incubated at 80°C for 5 minutes. 450 µl of 20% Biorad solution (Biorad, Hemel Hempstead, Herts. UK) was added to the alkali digested sample and incubated for 5 minutes at room temperature, after which time absorbance was measured as for standards.

6.2.6 Effect of dinitrophenol and nitrate on reduction of antimony V to antimony III by soil isolate UW-A and *Desulfotomaculm auripigmentum*

Desulfotomaculum auripgmentum ATCC 700205 was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and maintained on sulfate mineral medium solidified with 2% agar as described by Newman *et al.*[15]

Sulfate mineral medium (200 ml volume in 2 x 100 ml reaction vials) was inoculated with a turbid cell suspension of *D.auripigmentum* prepared by swabbing an agar plate.

After 5 days static incubation in the dark at 28°C, the cultures were combined and centrifuged (6000 rpm, 15 minutes) and the resultant pellet washed twice in fresh mineral medium containing no sulfate or antimony V. The cell pellet containing stationary phase cells was then re-suspended in this mineral medium to a final volume of 10 ml. 1 ml of this cell suspension was inoculated to 30 ml of mineral medium in 35 ml reaction vials sealed with PTFE-lined butyl rubber septa and aluminium crimp caps.

Mineral medium in reaction vials contained;

- potassium hexahydroxyantimonate as sole electron acceptor
- potassium hexahydroxyantimonate and magnesium sulfate as electron acceptors
- potassium hexahydroxyantimonate and 1 mmol.l^{-1} dinitrophenol (as uncoupler of oxidative phosphorylation from respiratory electron transport).

In all other respects, mineral medium was as described for the utilisation of a range of electron acceptors. Reaction vials were incubated statically in the dark at 28°C for 16 days, after which time total antimony and antimony V content were determined by HPLC-hydride generation-AFS analysis and culture supernatant was screened for the presence of methylantimony species by hydride generation-GC-AAS analysis.

Abiotic reduction of antimonate when pH and reduction potential of the surrounding environment is suitable can occur. At pH 7.0, reduction of antimony V to III requires a redox potential of +0.678V,[23] significantly higher than the redox potential generated by reduction of nitrate to nitrite and ammonia (+0.423 and +0.344V respectively),[24] or sulfate to sulfite and sulfide (-0.195 and -0.222V) [24]. Addition of antimony V to spent culture media containing sulfate or nitrate as respiratory electron acceptor would therefore likely result in the abiotic reduction of antimony V. For this reason the addition of antimony V to stationary phase cells of isolate UW-A and *D.auripigmentum* was made to washed cells in fresh media. Soil isolate UW-A was prepared in an identical manner except that potassium nitrate was substituted for magnesium sulfate as additional electron acceptor.

HPLC-hydride generation-AFS analysis of antimony

Analysis of antimony V content of culture supernatants was performed by HPLC-hydride generation-AFS as described above. Since inorganic antimony III did not elute using the column, total antimony concentration was determined by sample injection in the absence of guard and analytical columns.

Hydride generation-GC-AAS analysis

Hydride generated culture supernatants were analysed for the presence of methylantimony species by GC-AAS as described previously (section 3.2.6).

6.3 Results

6.3.1 Testing of soil isolates for antimony V to antimony III reducing capability

Of the eight bacterial isolates obtained from enrichment culture, six were found to react in a positive manner to the crude phosphomolybdic acid agar assay, i.e. the agar that six of the isolates were incubated upon turned blue when flooded with phosphomolybdic acid reagent, indicating the presence of antimony III. The colour of the phosphomolybdic acid reagent did not change colour when it came into contact with either of the agar plates of the remaining two isolates or the no biomass agar plate (Figure 6.1).

UV phosphomolybdic acid analysis of the soil isolates found to be positive in the crude agar assay, confirmed the bioreducing ability with respect to antimony. Supernatant from incubations of all six of the isolates tested accelerated the formation of the blue phosphomolybdate complex, indicating the presence of antimony III. One of the isolates from the urban wasteland soil was found to have the highest bioreducing capacity; up to 56.8 $\mu g.ml^{-1}$ antimony III was detected in culture supernatant after 6-weeks culture incubation (Table 6.1). Both soil isolates from the rural field location had lower antimony bioreducing capacity than the other isolates tested, and soil isolate Rural field B possessed the lowest antimony bioreducing capacity of all, with 17.0 $\mu g.ml^{-1}$ antimony III in detected culture supernatant. No antimony III was detected at any time in incubations lacking biomass, indicating that the reduction of antimony V to antimony III observed was biologically mediated. The ability of the isolates to bioreduce antimony was not linked to the presence of high quantities of antimony in the original soil sample. Samples from both urban wasteland and pond sediment had less than 0.1 $mg.g^{-1}$ (dry weight soil) antimony (as determined by ICP-MS - see Appendix III for data), whilst soil from the rural field (for which the lowest microbial bioreducing capacity was observed) contained the highest amount of antimony at 0.7 $mg.g^{-1}$ (dry weight soil).

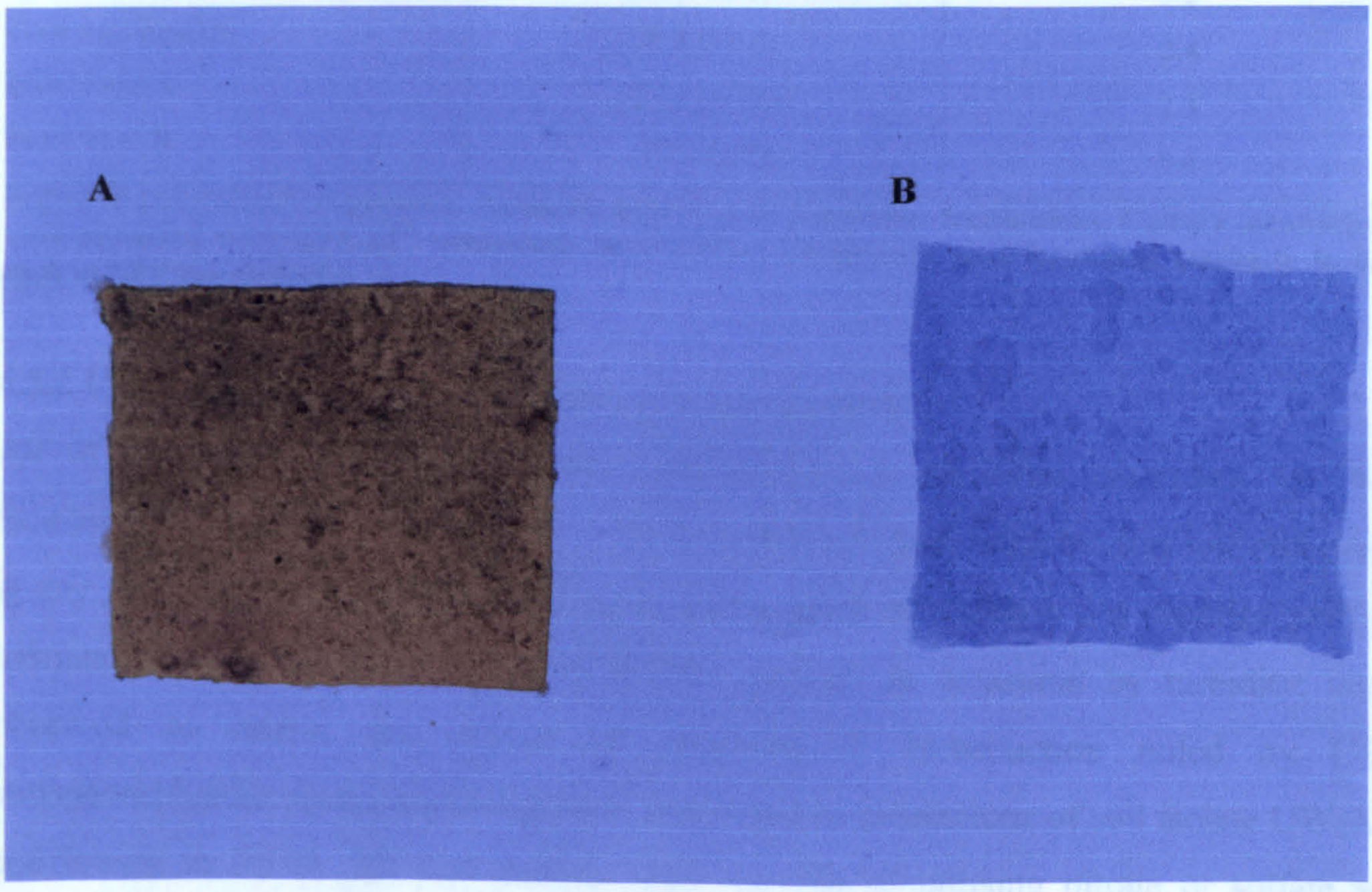


Figure 6.1 Agar from crude phosphomolybdic acid agar assay demonstrating colour change observed in the presence of antimony III. (A) Typical colour of agar containing only antimony V. (B) Typical colour of agar from incubation of a soil isolate possessing the capability to reduce antimony V to antimony III. (N.B. although not shown in photo, agar plates flooded with potassium antimony tartrate or antimony trioxide test solutions displayed an identical colouration to agar section B.

Table 6.1 Detection of antimony III in culture supernatant of soil isolates by phosphomolybdic acid assay.

Soil isolate	Amount of SbIII detected ($\mu g.ml^{-1}$)	% SbV converted
No biomass	0 (0)	0
Urban wasteland soil A	56.8 (4.8)	4.7
Urban wasteland soil B	34.6 (4.7)	2.8
Pond sediment A	38.5 (9.9)	3.2
Pond sediment B	28.8 (10.3)	2.4
Rural field A	27.8 (13.0)	2.3
Rural field B	17.0 (1.5)	1.4

Figure in parentheses represents standard deviation of 5 separate incubations. Culture incubations were supplied with 2.63 $g.l^{-1}$ potassium hexahydroxyantimonate, and incubated statically in the dark at 28°C for 6 weeks.

6.3.2 Utilisation of range of terminal electron acceptors

All of the soil isolates tested were demonstrated to be strict anaerobes, i.e. the facultative use of molcular oxygen as a respiratory electron acceptor was not observed at any time, nor was growth observed at any time in incubations not supplied with a terminal electron acceptor (Table 6.2). The growth, as measured by turbidity, was observed to mirror the degree of antimony V bioreduction noted by UV phosphomolybdic assay; highest growth was noted in incubations of soil isolate UW-A, and lowest in RF-B. All soil isolates were capable of utilising nitrate or sulfate as electron acceptor. Growth in incubations supplied with nitrate was more luxuriant than any incubations supplied with any other electron acceptor. This suggests that with the exception of soil isolate UW- B, the antimony bioreducing soil isolates are denitrifying microorganisms. The utilisation of molybdate and selenate as electron acceptor was observed in incubations of soil isolate UW- B and RF- B respectively. Isolates from the urban wasteland soil and one of the soil isolates from the rural field location were also found to possess the ability to grow in incubations supplied with sodium arsenate as electron acceptor. Bacterial respiration of selenate and arsenate is not unknown. The utilisation of selenate as terminal electron acceptor in anaerobic respiration has been previously reported,[14, 25], and three characterised strains of *D.auripigmentum* have been demonstrated to possess the ability to respire arsenate.[15]

Table 6.2 Utilisation of electron acceptors by soil isolates.

Soil isolate	Terminal electron acceptor						
	SeO_4^{2-}	MnO_2	AsO_4^{3-}	NO_3^-	$[Sb(OH)_6]^-$	SO_4^{2-}	O_2
UW- A	-	-	0.07	0.21	0.06	-	-
UW- B	-	0.03	0.06	-	0.05	0.09	-
PS- A	-	-	-	0.15	0.05	-	-
PS- B	-	-	-	0.14	0.04	-	-
RF-A	-	-	0.07	0.21	0.02	-	-
RF- B	0.03	-	-	0.18	0.02	-	-

- = no growth ; absorbance (600 nm) was measured against identical incubations lacking biomass inoculum; growth was not observed in incubations not supplied with a terminal electron acceptor. H_2 + lactate supplied as electron donor to all incubations. Soil isolates were isolated from urban wasteland (UW-A and UW-B), pond sediment (PS-A and PS-B) and a rural field (RF-A and RF-B).

6.3.3 Assay for presence of nitrate reductase in soil isolates

Nitrite (NO_2^-) was observed in culture supernatant of four of the soil isolates after 18 hours incubation in media supplied with nitrate as electron acceptor (Table 6.3). Of the two remaining soil isolates testing negative for the presence of nitrite, UW-A and B, soil isolate UW-A was found to have transformed the nitrate supplied to ammonium. The continued presence of nitrate and absence of nitrite in incubations of soil isolate Urban wasteland B was confirmed by continuation of the incubations, and re-analysis after 4 days. These data confirm the results obtained for soil isolate incubations tested for the utilisation of a range of electron acceptors. Turbidity measurements indicated that this soil isolate was the only one unable to utilise nitrate as an electron acceptor.

Table 6.3 Testing of antimony bioreducing soil isolates for nitrate reducing capability.

Soil isolate	Colour of medium after addition of sulphanilic acid and napthylamine	Subsequent colour change after addition of zinc dust.
UW- A	colourless	colourless
UW- B	colourless	red
PS- A	red	-
PS- B	red	-
RF-A	pink	-
RF- B	pink	-

- = not tested; red colouration of media after addition of sulphanilic acid and napthylamine indicates reduction of nitrate to nitrite. Continued absence of media colouration after addition of zinc dust indicates complete absence of nitrate due to its reduction to NH_4^+. Soil isolates were isolated from urban wasteland (UW-A and UW-B), pond sediment (PS-A and PS-B) and a rural field (RF-A and RF-B).

6.3.4 Assay of carbon source and antimonate utilisation and protein formation by soil isolate UW-A

The concomitant loss of carbon source (sodium lactate) and sole electron acceptor (potassium hexahydroxyantimonate) with the formation of protein (taken as a measure of biomass) was observed in incubations of soil isolate UW-A (Figure 6.2). The rates of utilisation of lactate and antimony V were highest during the period of exponential growth of soil isolate UW-A (as measured by protein concentration). This soil isolate was previously demonstrated to bioreduce antimony V to antimony III (section 6.3.1). The utilisation of nearly one third of the antimony V supplied was accompanied by the utilisation of just over half of the sodium lactate present, and protein formation amounting to 35.5 $\mu g.ml^{-1}$ (equivalent to a biomass concentration of approximately 59.2 $\mu g.g^{-1}$ (dry weight). No methylantimony species were detected in culture supernatant by hydride generation-GC-AAS at any time, indicating that the antimony III formed was probably inorganic in nature. Lactate and potassium hexahydroxyantimonate concentrations of no biomass incubations were not changed after 19 days incubation under identical conditions, nor was any protein detected in these samples, further confirming the biologically mediated nature of the antimony reduction by this soil isolate.

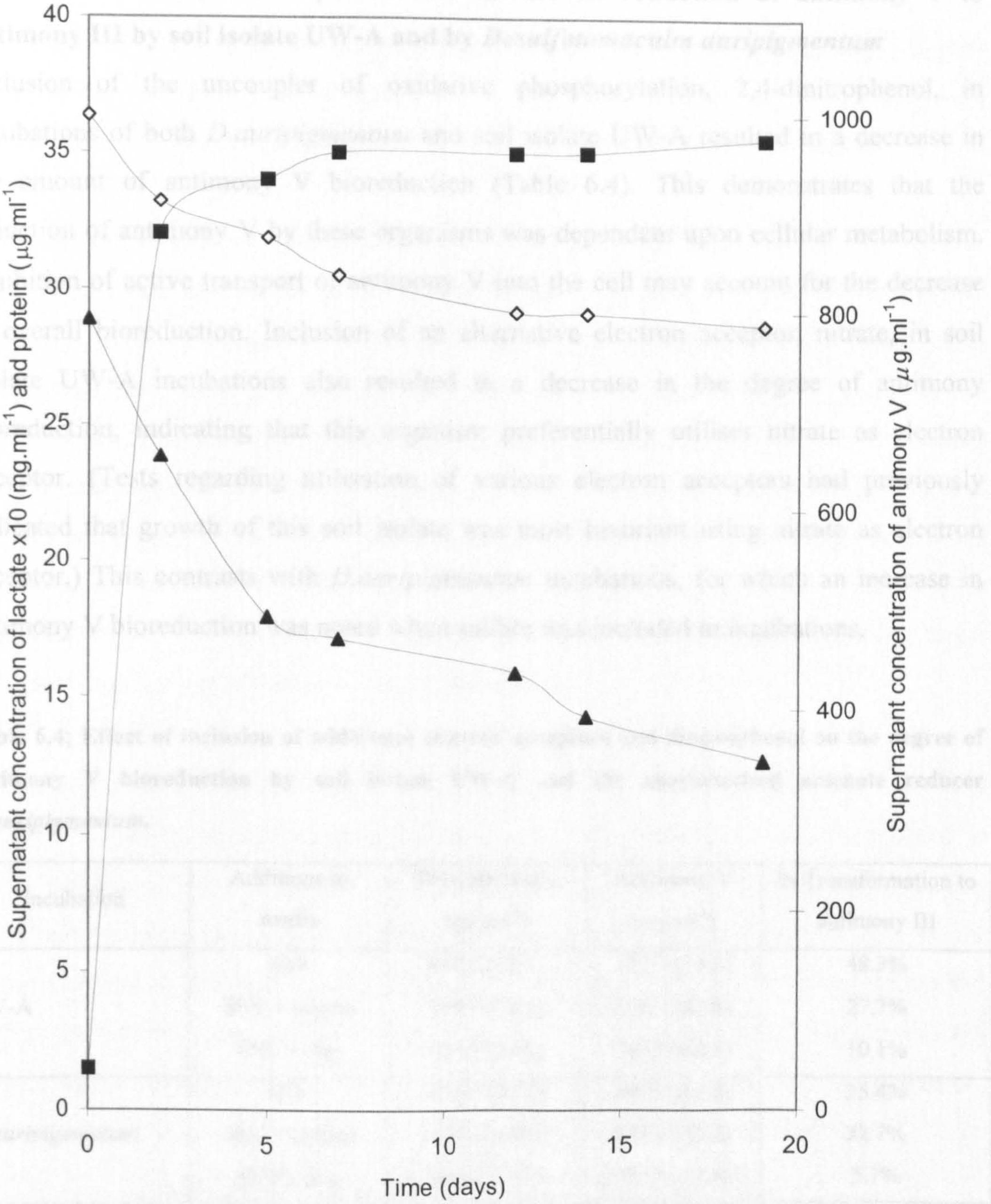


Figure 6.2 Profile of lactate (▲), antimony V disappearance (◇) and protein formation (■) in anaerobic incubations of soil isolate UW-A supplied with potassium hexahydroxyantimonate as electron acceptor, H_2 + sodium lactate as electron source and lactate as carbon source. Respective concentrations of lacate, protein and antimony V after 19-days incubation of "no biomass incubation"; [lactate] 2.86 (0.03) mg.ml^{-1}[protein] 0 (0) µg.ml^{-1}, [Antimony V] 1000.6 (13.2) µg.ml^{-1}. Maximum standard deviations (not shown on graph) observed (not shown on graph) were lactate 0.08 mg.ml^{-1}, protein 3.5 µg.ml^{-1} and antimony V 87.3 µg.ml^{-1}. Actual standard deviations are shown in Table AIII.8 (Appendix III). (Note that sodium lacate is presented on a ten-fold increased scale).

6.3.5 Effect of 2,4-dinitrophenol and nitrate on reduction of antimony V to antimony III by soil isolate UW-A and by *Desulfotomaculm auripigmentum*

Inclusion of the uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, in incubations of both *D.auripigmentum* and soil isolate UW-A resulted in a decrease in the amount of antimony V bioreduction (Table 6.4). This demonstrates that the reduction of antimony V by these organisms was dependent upon cellular metabolism. Inhibition of active transport of antimony V into the cell may account for the decrease in overall bioreduction. Inclusion of an alternative electron acceptor, nitrate, in soil isolate UW-A incubations also resulted in a decrease in the degree of antimony bioreduction, indicating that this organism preferentially utilises nitrate as electron acceptor. (Tests regarding utilisation of various electron acceptors had previously indicated that growth of this soil isolate was most luxuriant using nitrate as electron acceptor.) This contrasts with *D.auripigmentum* incubations, for which an increase in antimony V bioreduction was noted when sulfate was included in incubations.

Table 6.4; Effect of inclusion of additional electron acceptors and dinitrophenol on the degree of antimony V bioreduction by soil isolate UW-A and the characterised arsenate reducer *D.auripigmentum*.

Incubation	Additions to media	Total antimony ($\mu g.ml^{-1}$)	Antimony V ($\mu g.ml^{-1}$)	% Transformation to antimony III
UW-A	SbV	846.0 (32.1)	437.7 (33.8)	48.3%
	SbV + nitrate	910.7 (75.2)	658.3 (42.0)	27.7%
	SbV + dnp	881.7 (54.0)	793.0 (60.8)	10.1%
D.auripigmentum	SbV	925.0 (23.8)	690.0 (67.8)	25.4%
	SbV + sulfate	1315.7 (39.3)	886.0 (83.2)	32.7%
	SbV + dnp	970.3 (70.3)	915.0 (14.4)	5.7%

Figure in parentheses represents standard deviation of three separate incubations. Stationary phase cells of soil isolate UW-A and *D.auripigmentum* grown in media containing nitrate and sulfate as sole electron acceptor respectively (no antimony V) were washed and resuspended to equivalent turbidity in fresh media containing antimony V(potassium hexahydroxyantimonate), antimony V + sulfate or nitrate or antimony V + dnp (2,4-dinitrophenol). Incubation was continued for a further 16-days.

6.4 Discussion

A number of bacteria were isolated by enrichment culture from soil and sediment samples derived from varied habitats. Of the eight isolates obtained, six were demonstrated to possess the capability to bioreduce antimony V to antimony III.

A 5-fold difference was noted between the detection of antimony III by the UV phosphomolybdic assay and the calculation of antimony III levels from HPLC-hydride generation-AFS detection of antimony V and total antimony. Media composition differences are likely to account for much of this, since mineral and vitamin supplements were absent from the antimony mineral medium that was used for culture incubations analysed by the UV phosphomolybdic assay. The lag phase of the soil isolates when grown in antimony mineral medium was observed to be longer than when incubated in antimony-VM mineral medium. Addition of trace element and vitamin solutions to later experiments (i.e. those analysed by HPLC) decreased the lag phase and increased the overall amount of growth observed.

The ability of soil isolates UW-A, UW-B, PS-A, PS-B, RF-A and RF-B to bioreduce antimony V to an unknown inorganic antimony III compound was demonstrated to occur (section 6.3.1). It is reasonable to assume therefore, that the reduction in antimony V levels observed in cultures of soil isolate UW-A (section 6.3.4) is due to the transformation of antimony V to antimony III. It should be noted however that the full difference between total antimony and antimony V levels within an incubation will not equate solely to the amount of inorganic antimony III product within an incubation. Biosorption of antimony V (and antimony III) to cell surfaces will also occur.

Theoretical calculation of potassium hexahydroxyantimonate-biomass association (biosorption and uptake) using data obtained from *C.humicolus* studies (section 4.3.7) reveals that up to 109.4 $\mu g.g^{-1}$ potassium hexahydroxyantimonate could be associated with the bacterial biomass i.e. a total of 0.18 µg, compared to the removal of 218.6 $\mu g.ml^{-1}$ (6.65 mg) antimony V from culture media (antimony V as sole respiratory electron acceptor). In reality, the actual amount of biosorbed and non-transformed antimony V is likely to be significantly lower since (i) a constant positive rate of metal-biomass association, (ii) no subsequent biotransformation, and (iii) equivalent sorbing

capabilities of *C.humicolus* and soil isolate UW-A were assumed (fungi generally have significantly higher biosorbing capacities than bacteria).

The ability of soil isolate UW-A to reduce antimony V to an inorganic antimony III compound was demonstrated to be a biologically mediated process. No formation of antimony III was detected in culture incubations lacking biomass. The biomass dependency of the process was further demonstrated by the reduction in antimony V concentrations with concomitant removal of lactate (non-fermentable carbon source) and formation of biomass (as measured by protein concentration) in soil isolate UW-A cultures. Since antimony V was supplied as sole respiratory electron acceptor, these data suggest that the bioreduction of antimony V occurs through antimonate-respiration.

Despite the absence of respiratory electron acceptors such as sulfate and nitrate, and the stringent exclusion of oxygen from incubations, the possibility of alternative electron acceptors being scavenged from the medium, and dissimilatory antimony V bioreduction occurring cannot initially be discounted. The decrease that was observed in the level of antimony V bioreduction when nitrate was supplied as alternative electron acceptor to isolate UW-A however, clearly demonstrate the respiratory nature of antimony V bioreduction by soil isolate UW-A. Biomass levels in isolate UW-A cultures containing nitrate, or nitrate and antimony V were more luxuriant than those in cultures containing antimony V alone, demonstrating the preference of this soil isolate for nitrate respiration.

The enhancement of antimony V reduction in *D.auripigmentum* cultures containing both sulfate and antimony V compared to cultures containing antimony V alone indicate that antimony V reduction by this organism is most likely a biochemical and not a respiratory reduction. That is, energy derived from the reduction of antimonate is not utilised for growth by this organism. Possibly, alternative electron acceptors (not sulfate, oxygen or nitrate) were scavenged from the medium for respiration in incubations supplied with antimony V alone.

Inclusion of the uncoupler of oxidative phosphorylation, 2,4-dinitrophenol additionally demonstrated the dependency of antimony V bioreduction upon active cellular metabolism. Dinitrophenol dirupts proton translocation and the formation of a

transmembrane proton gradient across the inner membrane of the mitochondria [26]. This causes a cessation of oxidative phosphorylation and adenosine triosephosphate (ATP) synthesis by this route. Low levels of ATP may still be synthesised *via* glycolysis and the pentose phosphate pathways, however these are insufficient for maintenance of cellular metabolism. The reduced levels of antimony V bioreduction that was observed in cultures containing antimony V and dinitrophenol are compatible with the shut down of cellular metabolism because of a lack of ATP.

These data demonstrating biologically mediated formation of inorganic antimony III from inorganic antimony V substrate do not constitute the first report of antimony bioreduction; the formation of stibine from inorganic antimony III substrate (no change in valency state invoked) by the methanogen *Methanobacterium formicium* has recently been reported.[18] Furthermore, the formation of volatile methylantimony species from inorganic antimony V substrate [27]implies a capability for bioreduction of the metal if the Challenger mechanism for arsenic methylation is held to apply to antimony. The demonstration that growth and carbon source consumption were both linked with bioreduction of antimony V in incubations of soil isolate UW-A, however, demonstrates that for this isolate at least, antimony bioreduction is respiratory in nature. As yet, no reports of antimonate-respiring bacteria exist in the literature. In recent years, the number of bacteria known to possess the ability to respire "unusual" electron acceptors has expanded. Utilisation of Mn(IV), Se(VI), U(VI) and As(V) have all been reported.[9-12] Utilisation of many of these electron acceptors has been demonstrated to be widespread in the natural environment, occurring in both contaminated and pristine environments.[28, 29]

Recent reports of the dissimilatory reduction and respiration of antimony's element arsenic have indicated that this capability may be widespread in terms of both ecological niches and microbial species.[9, 15, 30, 31] Comparison of the free energy yielded from by dissimilatory reduction of arsenate and antimonate indicates the possibility for antimonate-respiration within the natural environment.

When the equations

$CH_3CHOHCOO^- + 2[Sb(OH)_6]^- \rightarrow CH_3COO^- + 2[Sb(OH)_4]^- + CO_2 + 3H_2O \quad \Delta G^\circ = -203 kJ.mol^{-1}$

$CH_3CHOHCOO^- + 2HAsO_4^{2-} \rightarrow CH_3COO^- + 2HAsO_3^{2-} + CO_2 + 3H_2O \quad \Delta G^\circ = -172 kJ.mol^{-1}$

are considered, it can be seen that antimonate is a better oxidant than arsenate and that more free energy is yielded when coupled to the oxidation of lactate. The bioreduction of $[Sb(OH)_6]^-$ may not necessarily terminate at the species $[Sb(OH)_4]^-$, but could theoretically progress through to the formation of stibine (SbH_3), contributing to the detection of stibine reported in landfill gases,[32, 33] or elemental antimony (Sb^0). These reactions yield respective free energies of -60 $kJ.mol^{-1}$ and -207 $kJ.mol^{-1}$ when coupled to the oxidation of lactate. There seems little thermodynamic reason therefore why the potential for antimonate-respiration should not occur, and perhaps be relatively widespread within anaerobic environments.

For all but one of the soil isolates able to bioreduce antimony, nitrate appeared to be the preferred electron acceptor. This observation can be explained on thermodynamic grounds since reduction of nitrate to nitrite (-231.3 $kJ.mol^{-1}$) and the reduction of nitrate to ammonium (-245.0 $kJ.mol^{-1}$) both yield more energy than the corresponding reduction of $[Sb(OH)_6]^-$ to $[Sb(OH)_4]^-$. The observation that soil isolate UW-B preferred sulfate to antimonate is however paradoxical since the reduction of sulfate to hydrogen sulphide yields only –89 $kJ.mol^{-1}$ free energy. Clearly more factors than thermodynamic considerations impact on the choice of utilised electron acceptor. Oremland *et al* [14] noted similar anomalies for the growth of an anaerobic selenate-respiring bacterium. Growth of a Gram-negative *Vibrio* sp. was reported to be significantly more favourable utilising nitrate as an electron acceptor as opposed to selenate. Based on various toxicological studies Oremland *et al.* suggested that the reason for this thermodynamic paradox was due to the toxicity of selenium to growing cells [14]. In the absence of toxicological data for the soil isolates studied here it is impossible to definitively say that the toxicity of antimonate prohibits or limits its utilisation as an electron acceptor. Given the known toxicity of certain antimony compounds however [34, 35] and the data obtained for both antimony V and III species regarding fungal toxicology presented earlier (Sections 4.3.2, 5.3.3 & 5.3.6) it is tempting to consider this possibility. Certainly, the loading levels of antimonate in these experiments are several orders of

magnitude higher than that found naturally in the environment. Sloof *et al.* [36] reported global concentrations of antimony to vary between 0.15 and 20 $mg.kg^{-1}$. To follow respiratory utilisation of antimonate required used of millimolar amounts. It therefore follows that in natural environments the amount of carbon mineralised through dissimilatory antimonate reduction is likely to be low, perhaps even negligible. The possibility exists though, that the capability to grow through utilisation of antimonate as an electron acceptor may be advantageous in certain environments, which display elevated antimony concentration and deficient in alternative electron acceptors, for example semiconductor disposal facilities or mine tailings.

References for Chapter 6

1. C. Cervantes, J. Guangyong, J. L. Ramirez, and S. Silver, Resistance to arsenic compunds in microorganisms. *FEMS Microbiology Reviews* **15**, 355 (1994).
2. B. P. Rosen, Bacterial resistance to heavy metals and metalloids. *Journal of Biological Inorganic Chemistry* **1**, 273 (1996).
3. A. O. Summers, *in* Antimicrobial Drug Resistance L. E. Bryan, Vol. 345. Academic Press, 1984.
4. G. Ji and S. Silver, Bacterial resistance mechanisms for heavy metals of environmental concern. *Journal of Industrial Microbiology* **14**, 1995).
5. J. J. Wrench and R. F. Addison, Reduction, methylation and Incorporation of arsenic into lipids by the marine phytoplankton *Dunaliella tertiolecta. Canadian Journal of Fish and Aquatic Science* **38**, 518 (1981).
6. M. Slekovec, W. Goessler, and K. J. Irgolic, Inorganic and organic arsenic compounds in Slovenian mushrooms: comparison of arsenic-specific detectors for liquid chromatography. *Chemical Speciation and Bioavailability* **11**, 115 (1999).
7. F. G. Ferris, W. Shotyk, and W. S. Fyfe, *in* Metal Ions and Bacteria T. J. Berveridge and R. J. Doyle, Vol. John Wiley and Sons, New York, 1989.
8. P. Levi and A. Linkletter, *in* Metals and Microorganisms M. N. Hughers and R. K. Poole, Vol. 305. Chapman and Hall, London, 1989.
9. D. Ahmann, L. R. Roberts, L. R. Krumholz, and F. M. M. Morel, Microbe grows by reducing arsenic. *Nature* **371**, 750 (1994).
10. P. R. Dowdle, A. M. Laverman, and R. S. Oremland, Bacterial dissimilatory reduction of arsenic (V) to arsenic (III) in anoxic sediments. *Applied and Environmental Microbiology* **62**, 1664 (1996).
11. D. R. Lovely, E. J. Phillips, and D. J. Lonergan, Dissimilatory (Fe)III and Mn(IV) reduction. *Microbial Reviews* **55**, 263 (1991).
12. K. H. Nealson and D. A. Saffarini, Iron and manganese in anaerobic respiration: environmental significance, physiology and regulation. *Annual Review of Microbiology* **48**, 311 (1994).
13. F. Widdel and N. Pfennig, Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. II. Incomplete oxidation of propionate by *Desulfobubus propionicus* gen nov., sp. nov. *Archives of Microbiology* **131**, 360 (1982).
14. R. S. Oremland, J. S. Blum, C. W. Culbertson, P. T. Visscher, L. G. Miller, P. Dowdle, and F. Strohmaier, Isolation, growth, and metabolism of an obligately anaerobic, selenate-respiring bacterium, Strain SES-3. *Applied and Environmental Microbiology* **60**, 3011 (1994).
15. D. K. Newman, E. K. Kennedy, J. D. Coates, D. Ahmann, D. J. Ellis, D. R. Lovley, and F. M. M. Morel, Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. *Archives of Microbiology* **168**, 380 (1997).
16. J. M. Macy, S. Rech, G. Auling, M. Dorsch, E. Stackebrandt, and L. I. Sly, *Thauera selenatis* gen. nov., sp. nov., a memeber of the beta subclass of *Proteobacteria* with a novel type of anaerobic respiration. *International Journal of Systematic Bacteriology* **43**, 135 (1993).

17. H. Gürleyük, V. Van Fleet-Stalder, and T. G. Chasteen, Confirmation of the biomethylation of antimony compounds. *Applied Organometallic Chemistry* **11**, 471 (1997).

18. K. Michalke, E. B. Wickenheiser, M. Mehring, A. V. Hirner, and R. Hensel, Production of volatile derivatives of metal(loid)s by microflora involved in anaerobic digestion of sewage sludge. *Applied and Environmental Microbiology* **66**, 2791 (2000).

19. S. Honschopp, N. Brunken, A. Nehrkorn, and H. J. Breunig, Isolation and characterisation of a new arsenic methylating bacterium from soil. *Microbiological Research* **151**, 37 (1996).

20. D. Sicilia, S. Rubio, and D. Perez-Bendito, Kinetic determination of antimony(III) based on its accelerating effect on the reduction of 12-phosphomolybdate by ascorbic Acid in a micellar medium. *Analytical Chemistry* **64**, 1490 (1992).

21. D. K. Newman, T. J. Beveridge, and F. M. M. Morel, Precipitation of arsenic trisulfide by *Desulfotomaculum auripigmentum*. *Applied and Environmental Microbiology* **63**, 2022 (1997).

22. J. Lintschinger, I. Koch, S. Serves, J. Feldmann, and W. R. Cullen, Determination of antimony species with high-performance liquid chromatography using element specific detection. *Fresenius Journal of Analytical Chemistry* **359**, 484 (1997).

23. P. J. Craig and F. E. Brinckman, *in* Organometallic compounds in the environment. Principles and reactions P. J. Craig, Vol. 1. Longman Group Limited, Bath, UK, 1986.

24. Lecture No. 8. Eh-pH diagrams II. 2001, p. 2001).

25. J. M. Macy and S. Lawson, Cell yield (Y_M) of *Thauera selenatis* grown anaerobically with acetate pul selenate or nitrate. *Archives of Microbiology* **160**, 295 (1993).

26. M. W. King, The Medical Biochemistry Page: Oxidative Phosphorylation. 2001, p. King, M. W., 1998).

27. R. O. Jenkins, P. J. Craig, W. Goessler, D. Miller, N. Ostah, and K. J. Irgolic, Biomethylation of inorganic antimony compounds by an aerobic fungus: *Scopulariopsis brevicaulis*. *Environmental Science and Technology* **32**, 882 (1998).

28. R. S. Oremland, N. A. Steinberg, T. S. Presser, and L. G. Miller, *In situ* bacterial selenate reduction in the agricultural drainage systems of western Nevada. *Applied and Environmental Microbiology* **57**, 615 (1991).

29. N. A. Steinberg and R. S. Oremalnd, Dissimilatory selenate reduction potentials in a diversity of sediment types. *Applied and Environmental Microbiology* **56**, 3550 (1990).

30. D. Ahmann, L. R. Krumholz, H. F. Hemond, D. R. Lovely, and F. M. M. Morel, Microbial mobilization of arsenic from sediments of the Aberjona watershed. *Environmental Science and Technology* **31**, 2923 (1997).

31. A. M. Laverman, J. S. Blum, J. K. Schaefer, E. J. P. Phillips, D. R. Lovley, and R. S. Oremland, Growth of Strain SES-3 with arsenate and other diverse electron acceptors. *Applied and Environmental Microbiology* **61**, 3556 (1995).

32. J. Feldmann and A. V. Hirner, Occurence of volatile metal and metalloid species in landfill and sewage gases. *International Journal of Environmental and Analytical Chemistry* **60**, 339 (1995).

33. J. Feldmann, R. Grümping, and A. V. Hirner, Determination of volatile metal and metalloid compounds in gases from domestic waste deposits with GC/ICP-MS. *Fresenius Journal of Analytical Chemistry* **350**, 228 (1994).

34. K. A. Winship, Toxicity of antimony and its compounds. *Adverse Drug Reaction and Acute Poisoning Reviews* **2**, 67 (1987).

35. A. Leonard and G. B. Gerber, Mutagenicity, carcinogenicity and teratogenicity of antimony compounds. *Mutation Research* **366**, 1 (1996).

36. W. Sloof, P. F. H. Bont, J. M. Hesse, and B. Loos, Antimony and antimony compounds. RIVM (National Institute of Public Health and Environmental Protection), (1992).

7 General Conclusion

As stated at the outset of the thesis, data regarding the occurrence and speciation of antimony within environmental compartments is not extensive, and little is known about the geochemical cycling of the element and the role microorganisms play in this cycle. This contrasts the state of the literature regarding antimony's element arsenic for which detailed mechanisms of microbial biomethylation, bioreduction and bio-oxidation have been elucidated.

The demonstration of antimony biomethylation by five fungi (*Cryptococcus humicolus*, *Candida boidinii*, *Candida tropicalis*, *Geotrichum candidum*, and *Saccharomyces cerevisiae*) and five anaerobic bacteria (*Clostridium acetobutylicum*, *Clostridium butyricum*, *Clostridium cochlearium*) and two clostridial isolates from soil enrichment culture has significantly extended the number of microorganisms known to methylate inorganic antimony. Of these species, only *C.humicolus* and *C.cochlearium* have been demonstrated previously to possess a metal biomethylating capability; *C.humicolus* for arsenic,[1, 2] and *C.cochlearium* for mercury.[3] The fungus *Scopulariopsis brevicaulis* was, for the first time shown, to produce involatile methylantimony species from *both* inorganic III and V valency antimony compounds. Whilst the formation of volatile methylantimony from an inorganic antimony V compound by this organism has been demonstrated [4] it has been suggested that *S.brevicaulis,* and fungi in general, are incapable of biomethylating antimony in the V valency state since uptake of such compounds is prohibited.[5] The demonstration of an antimony V biomethylating capability for *C.humicolus* and *S.brevicaulis*, coupled with the demonstration of a metabolism-dependant uptake of the antimony V compound potassium hexahydroxyantimonate for *C.humicolus* clearly demonstrates that this view is erroneous.

In contrast to *S.brevicaulis*,[4] the capability of *C.humicolus* to biomethylate antimony was demonstrated not to be a feature of the linear phase of growth. Inhibition of protein synthesis at the beginning of the stationary phase of growth resulted in the almost complete absence of biomethylation demonstrating that the necessary enzymes must be formed at a later time point. The antimony biomethylating capability of *C.humicolus* was however almost fully developed by the mid-stationary phase. These data underline species differences in the actual processing of antimony.

The mechanism of antimony biomethylation in the fungi and bacteria tested has shown similarities in that there appears to be a progression of mono- through di- to trimethylated species. This was particularly apparent in *Clostridium* sp. incubations for which monomethylantimony was detected as the sole involatile methylantimony species in early stages of incubation. Furthermore, the percentage contribution of monomethylated antimony species to the total amount of involatile methylantimony detected decreased throughout the incubation period. These data are consistent with a trimethylantimony compound being the final product of antimony biomethylation with mono- and dimethylated species appearing transiently in culture supernatants as intermediates of the antimony biomethylation pathway. The transformation of involatile di- to trimethylated antimony species was seen to be a rate-limiting step to a greater or lesser degree in the fungal species. In contrast, the accumulation of involatile dimethylantimony was not observed at any time in culture supernatants of any of the *Clostridium* spp. tested, nor were dimethylated antimony species detected alongside trimethylated forms. This may be a factor of antimony loading of the methylation pathway, since it was clearly demonstrated that the concentration of dimethylantimony species was directly correlated to the total amount of involatile methylantimony species detected in *C.humicolus* and *S.brevicaulis* incubations. Lower methylation efficiency and overall loading of the antimony pathway in *Clostridium* may mean that the methyltransferase responsible for the di- to tri- methylantimony transformation is not saturated. These data are compatible with the mechanism of antimony biomethylation being analogous to that described for arsenic.[6] The detection of involatile antimony species was always greater than the detection of volatile forms in incubations of all fungal and bacterial species tested. A phenomenon that has also been demonstrated for aerobic cultures of the fungus *S.brevicaulis*.[5]

Internal cellular accumulation of methylantimony species was shown to occur. Lysis of *C.humicolus* cells after 6-days incubation resulted in not only significantly higher amounts of methylantimony species in culture supernatants compared to non-lysed incubations (170-fold higher), but also significantly higher levels than normally observed in culture supernatants after 19-days incubation (9-fold higher) (at the 95% confidence level). The higher proportion of internalised mono- and dimethylated antimony species compared to supernatant levels indicates not only that a

trimethylantimony species is the end product of the biomethylation pathway, but that it is also the primary exported species and passive diffusion of the lower methylated species through the cellular membrane does not occur to a significant degree.

There are several reports of antimony biomethylation by undefined communities of bateria grown under anaerobic conditions.[7-9] It could be construed on the basis of these reports that antimony biomethylation capability may be linked to an ability to generate low redox potential environments. Indeed, the requirement for an aerobic atmosphere (at least on a microscale) and the presence of reducing conditions have been listed as some of the most important criteria for a biomethylating capability.[10] Analysis of the redox potential generated during typical *Clostridium* spp. incubation revealed no evidence for such a correlation. This suggests that the formation of methylantimony compounds by mixed communities of bacteria under anaerobic conditions is determined by the presence of individual species with specific antimony biomethylating capability rather than by the overall environmental conditions generated by mixed communities. Furthermore, the detection of involatile antimony species in culture supernatants of pure clostridial isolates from soil enrichment cultures shown to volatilise antimony, indicates that mixed community functioning is not an obligate requirement for antimony biomethylation by undefined soil/sediment enrichment conditions that promote the growth of clostridia.[8, 9]

Biomethylation efficiencies from inorganic antimony substrate were extremely low for all microorganisms tested. For example, *C.humicolus*, the most productive species in terms of formation of methylantimony compounds, transformed less than 0.1% of inorganic antimony III substrate to methylated species. This is up to 100-fold less than the biomethylation efficiency observed from arsenic substrate. Comparison of uptake efficiencies of arsenic and antimony by this microorganism revealed that differences in rates of metal-biomass association of the metals do not account fully for this variation. These data indicate that *all mechanisms* of cellular processing of antimony are not necessarily analogous and occur at a reduced level to those processes that exist for arsenic.

With regard to biomethylation of the two metals, the interrelationship between arsenic and antimony biomethylation was further elucidated by the demonstration of up-

regulation of antimony biomethylation upon pre-incubation of *C.humicolus* cells in media containing inorganic arsenic. The induction of antimony biomethylation by arsenic further demonstrates the enzymatic nature of the process and may suggest that the enzymes of the arsenic biomethylation pathway are the likely catalysts for the biomethylation of antimony. Indeed the low efficiency of antimony biomethylation indicates that this is most likely a fortuitous process.

In addition to the detection of volatile methylantimony species, stibine (SbH_3) was identified in the headspace gases of *C.humicolus, S.brevicaulis* and *S.cerevisiae.* The gas was not identified in fully aerobic incubations, but only when biomass was incubated anaerobically after a short aerobic incubation period. Furthermore, biomass concentration was required to attain detectable levels of volatile antimony species. Such conditions impose great stress upon cellular physiology, and whilst they demonstrate the bioreducing capability of these fungi, the extrapolation of such data to real-life environmental situations is questionable. Andrewes *et al.* [5]similarily reported the low level of detection of stibine in the headspace gases of fully aerobic nine litre cultures of *S.brevicaulis* that were sealed for up to 24 hours prior to gas sampling. Calculations demonstrate however that oxygen limitation would likewise occur under such a regime leading to the imposition of physiological stress.

The detection of higher levels of stibine formation from antimony V than antimony III substrates by *C.humicolus* and *S.brevicaulis* suggests a capability for antimony bio-oxidation by these species. That is, inorganic antimony III is first oxidised to antimony V before reduction to stibine. The capability of *S.brevicaulis* to oxidise antimony has been suggested before, although supporting data were not presented.[5]

Further bioreduction of antimony was demonstrated by the ability of a number of anaerobic Gram positive cocci isolated from soil and sediment samples to form antimony III from inorganic antimony V substrate supplied as sole electron acceptor. The demonstration of dissimilatory reduction of antimony coupled to lactate oxidation constitutes the first report of bacteria able to derive energy for growth and the maintenance of biomass from the reduction of antimony. Clearly such a capability could be advantageous in environmental situations when antimony concentrations are elevated and other more common electron acceptors are deficient. In contrast to the preferred use

of nitrate as an electron acceptor by most of the soil isolates, the higher growth yield of soil isolate UW- B when grown with sulfate indicates that higher energy yield from the coupling of the reduction of a metal to the carbon source oxidation is not the sole deciding factor in choice of electron acceptor. Factors such as cellular toxicity also likely impact.[11] The demonstration of antimonate-respiration adds this metal to the increasing list of known "unusual" electron acceptors: arsenic, uranium, iron, manganese and selenium.[12-15] *Desulfotomaculum auripigementum*, known to be capable of arsenate-respiration [16]was also found to be capable of antimony bioreduction. In contrast to the soil isolates obtained, reduction of antimonate by this organism was not however dissimilatory in nature.

Clearly microbial interactions with the metal antimony in its various forms are extensive and varied. Whilst this element is considered to be relatively toxic and non-essential,[17] the involvement of the metal in metabolic processes in certain situations has been demonstrated. Likewise the contribution of microorganisms to the biogeochemical cycling of the metal through various biotransformations - methylation, reduction and oxidation appears extensive. The formation of volatile and involatile methylantimony species from inorganic antimony substrate of both valency forms for example, increases the environmental mobility of the metal and will likewise influence the toxicity and bioaccumulation of the element. The toxicity of many of these antimony compounds remains to be clearly established. The extrapolation of toxicological data for arsenic compounds, however, and the extensive and increasing anthropogenic usage of antimony compounds may yet raise new public health issues and considerations with regard to the management of antimony containing wastes.

References for Chapter 7

1. D. P. Cox and M. Alexander, Production of trimethylarsine gas from various arsenic compounds by three sewage fungi. *Bulletin of Environmental Contamination & Toxicology* **9**, 84 (1973).
2. W. R. Cullen, C. L. Froese, A. Lui, M. B. C., D. J. Patmore, and M. Reimer, The aerobic methylation of arsenic by microorganisms in the presence of L-methionine-*methyl-d_3*. *Applied Organometallic Chemistry* **139**, 62 (1977).
3. H. S. Pan-Hou and N. Imura, Physiological role of mercury methylation in *Clostridium cochlearium* T-2C. *Bulletin of Environmental Contamination and Toxicology* **29**, 290 (1982).
4. R. O. Jenkins, P. J. Craig, W. Goessler, D. Miller, N. Ostah, and K. J. Irgolic, Biomethylation of inorganic antimony compounds by an aerobic fungus: *Scopulariopsis brevicaulis*. *Environmental Science and Technology* **32**, 882 (1998).
5. P. Andrewes, W. R. Cullen, J. Feldmann, I. Koch, P. E., and K. J. Reimer, The production of methylated organoantimony compounds by *Scopulariopsis brevicaulis*. *Applied Organometallic Chemistry* **12**, 827 (1998).
6. F. Challenger, Biological methylation. *Chemical Reviews* **36**, 315 (1945).
7. H. Gürleyük, V. Van Fleet-Stalder, and T. G. Chasteen, Confirmation of the biomethylation of antimony compounds. *Applied Organometallic Chemistry* **11**, 471 (1997).
8. P. N. Gates, H. A. Harrop, J. B. Pridham, and B. Smethurst, Can microorganisms convert methylated antimony trioxide or potassium antimonyl tartrate to methylated stibines? *Science of the Total Environment* **205**, 215 (1997).
9. R. O. Jenkins, P. J. Craig, D. P. Miller, L. C. A. M. Stoop, N. Ostah, and T.-A. Morris, Antimony biomethylation by mixed cultures of micro-organisms under anaerobic conditions. *Applied Organometallic Chemistry* **12**, 449 (1998).
10. A. V. Hirner, U. M. Grueter, and J. Kresimon, Metal(oid)organic compounds in contaminated soil. *Fresenius Journal of Analytical Chemistry* **368**, 263 (2000).
11. R. S. Oremland, J. S. Blum, C. W. Culbertson, P. T. Visscher, L. G. Miller, P. Dowdle, and F. Strohmaier, Isolation, growth, and metabolism of an obligately anaerobic, selenate-respiring bacterium, Strain SES-3. *Applied and Environmental Microbiology* **60**, 3011 (1994).
12. D. Ahmann, L. R. Roberts, L. R. Krumholz, and F. M. M. Morel, Microbe grows by reducing arsenic. *Nature* **371**, 750 (1994).
13. D. R. Lovely, E. J. Phillips, and D. J. Lonergan, Dissimilatory (Fe)III and Mn(IV) reduction. *Microbial Reviews* **55**, 263 (1991).
14. P. R. Dowdle, A. M. Laverman, and R. S. Oremland, Bacterial dissimilatory reduction of arsenic (V) to arsenic (III) in anoxic sediments. *Applied and Environmental Microbiology* **62**, 1664 (1996).
15. K. H. Nealson and D. A. Saffarini, Iron and manganese in anaerobic respiration: environmental significance, physiology and regulation. *Annual Review of Microbiology* **48**, 311 (1994).
16. D. K. Newman, E. K. Kennedy, J. D. Coates, D. Ahmann, D. J. Ellis, D. R. Lovley, and F. M. M. Morel, Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. *Archives of Microbiology* **168**, 380 (1997).

17. L. Friberg, G. F. Nordberg, and V. B. Vouk, Handbook on the toxicology of metals. Volume II: Specific metals. *Elsevier Science Publisher B.V.* 27 (1986).

APPENDIX I DEMETHYLATION ADJUSTMENT OF GC-AAS DATA

I.1 Introduction

Dismutation, demethylation and rearrangement are all words that are used in the literature to describe the problem of the loss of methyl groups when a methylated metal species is derivatised under non-optimal conditions. This means that hydride generation tends to produce more than one product from a single analyte, e.g. hydride generation of a trimethylated antimony compound can, in addition to trimethylstibine, result in the presence of stibine, monomethylstibine, and dimethylstibine.[1, 2] This has been found to be a particular problem with antimony speciation in environmental samples, and lack or appropriate consideration to this problem has resulted in the findings of some groups being called into question.[3, 4] Although the phenomenon can be limited by manipulation of hydride generation reaction conditions, it cannot always be fully eliminated.

I.2 Method

The degree of demethylation occurring upon sodium borohydride derivatisation of methylantimony species present in culture supernatants was corrected for by comparison to the degree of demethylation observed upon derivatisation of trimethylantimony chloride standards run on the day of analysis. That is to say, the contribution of the demethylation of higher methylated antimony species was assessed and correction was subsequently made for the demethylation to lower methylated species. Since no mono or dimethylstibine standards were available, the demethylation of di- and monomethylantimony compounds was assumed to be identical to that observed for the mono- and dimethylantimony species generated upon sodium borohydride derivatistion of trimethylantimony dichloride. i.e. the trimethylstibine peak generated by derivatisation of trimethylantimony chloride was ignored for the calculation of the demethylation of a dimethylantimony compound. Trimethylantimony chloride standards were prepared in aqueous solution rather than fresh or spent incubation media since no significant difference (at the 95% confidence level) in degree of demethylation or amount detected was observed using the different reaction media (Table I.1).

Table I.1 Derivatisation of 50 ng.ml^{-1} trimethylantimony chloride in different reaction media.

Reaction media	Amount detected (ng.ml^{-1})			
	Sb_{inorg}	MeSb	Me_2Sb	Me_3Sb
Water	0 (0)	0.8 (0.2)	7.5 (0.8)	41.7 (2.4)
Fresh YM medium	0 (0)	1.0 (0.4)	7.4 (1.1)	41.6 (2.9)
Spent YM medium	0 (0)	0.9 (0.2)	6.9 (1.1)	42.2 (2.3)

Derivatisation of trimethylantimony dichloride was performed as described for GC-AAS analysis; figure in parentheses are standard deviations based on three replicate analyses; there was no statistical difference between the means at the 95% confidence level.

TRIMETHYLANTIMONY

$$Me_3Sb_{corrected} = Me_3Sb_{measured} + (Me_3Sb_{measured} \times (\frac{Me_2Sb_{std} + MeSb_{std} + SbH_{3\,Std}}{Me_3Sb_{std}}))$$

DIMETHYLANTIMONY

Calculation of amount of dimethylantimony arising by demethylation of trimethylantimony species

$$Me_2Sb_{Tri} = \frac{Me_3Sb_{corrected} \times Me_2Sb_{std}}{Me_3Sb_{total\ std}}$$

Calculation of amount of dimethylantimony species present in sample

$$Me_2Sb_{Di} = Me_2Sb_{measured} - Me_2Sb_{Tri}$$

Correction of dimethylantimony for amount lost by demethylation to lower methylated species

$$Me_2Sb_{corrected} = Me_2Sb_{Di} + (\frac{Me_2Sb_{Di} \times (MeSb_{std} + SbH_{3\,Std}))}{Me_2Sb_{std}}$$

MONOMETHYLANTIMONY

Calculation of amount of monomethylantimony arising by demethylation of trimethylantimony species

$$MeSb_{Tri} = \frac{Me_3Sb_{corrected} \times MeSb_{std}}{}$$

$Me_3Sb_{\text{total std}}$

Calculation of amount arising by demethylation of dimethylantimony species

$$MeSb_{Di} = \frac{Me_2Sb_{corrected} \times MeSb_{std}}{Me_2Sb_{\text{total std}}}$$

Calculation of amount of monomethylantimony species present in sample

$$MeSb_{Mono} = MeSb_{measured} - MeSb_{Tri} - MeSb_{Di}$$

Correction of monomethylantimony for amount lost by demethylation to lower methylated species

$$MeSb_{corrected} = MeSb_{Mono} + \left(\frac{MeSb_{Mono} \times SbH_{3\_std}}{MeSb_{std}}\right)$$

Key

$Me_3Sb\,(Me_2Sb\ Me_2Sb\ SbH_3)_{measured}$

concentration of methylantimony species before demethylation correction

$Me_3Sb\,(Me_2Sb\ Me_2Sb\ SbH_3)_{corrected}$

concentration of methylantimony species after demethylation correction

$Me_3Sb\,(Me_2Sb\ Me_2Sb\ SbH_3)_{std}$

peak area of methylated species generated by hydride generation of trimethylantimony dichloride standard

$Me_3Sb_{\text{total std}}$

total peak area generated by hydride generation of trimethylantimony dichloride standard

$Me_2Sb_{\text{total std}}$

total peak area of dimethylated, monomethylated antimony species and stibine generated by hydride generation of trimethylantimony dichloride standard

$Me_2Sb\,(MeSb)_{Tri}$

amount of methylantimony species arising through demethylation of trimethylated antimony

Me_2Sb_{Di}

measured amount of dimethylantimony species corrected for demethylated species from trimethylantimony

$MeSb_{Di}$

amount of mnonomethylantimony species arising through demethylation of dimethylated antimony

$MeSb_{Mono}$

measured amount of monomethylantimony species corrected for demethylated species from di- and trimethylantimony

Example 1:

using data obtained for analysis of 50 $ng.ml^{-1}$ trimethylantimony chloride standard (reaction media = water), for which trimethylated species are the only methylantimony species supplied to the derivatisation reaction.

	Sb_{inorg}	MeSb	Me_2Sb	Me_3Sb
mean supernatant concentration ($ng.ml^{-1}$)	0	0.8	7.5	41.7
peak area	0	3.5	32.7	181.8

TRIMETHYLANTIMONY

$$Me_3Sb_{corrected} = 41.7 + \left(41.7 \times \frac{32.7 + 3.5 + 0}{181.8}\right)$$

$Me_3Sb_{corrected} = 50.0\ ng.l^{-1}$

DIMETHYLANTIMONY

Calculation of amount of dimethylantimony arising by demethylation of trimethylantimony species

$$Me_2Sb_{Tri} = \frac{50.0 \times 32.7}{218}$$

$Me_2Sb_{Tri} = 7.5\ ng.l^{-1}$

Calculation of amount of dimethylantimony species present in sample

$Me_2Sb_{Di} = 7.5 - 7.5$

$Me_2Sb_{Di} = 0\ ng.l^{-1}$

Correction of dimethylantimony for amount lost by demethylation to lower methylated species

$$Me_2Sb_{corrected} = 0 + \frac{0 \times (3.5 + 0)}{32.7}$$

$Me_2Sb_{corrected} = 0\ ng.l^{-1}$

MONOMETHYLANTIMONY

Calculation of amount of monomethylantimony arising by demethylation of trimethylantimony species

$$MeSb_{Tri} = \frac{50.0 \times 3.5}{218}$$

$MeSb_{Tri} = 0.8\ ng.l^{-1}$

Calculation of amount arising by demethylation of dimethylantimony species

$$MeSb_{Di} = \frac{0 \times 3.5}{8.3}$$

$MeSb_{Di} = 0\ ng.l^{-1}$

Calculation of amount of monomethylantimony species present in sample

$MeSb_{Mono} = 0.8 - 0.8 - 0$

$MeSb_{Mono} = 0\ ng.l^{-1}$

Correction of monomethylantimony for amount lost by demethylation to lower methylated species

$$MeSb_{corrected} = 0 + \left(\frac{0 \times 0}{3.5}\right)$$

$MeSb_{corrected} = 0\ ng.l^{-1}$

Example 2:

using data obtained for typical analysis of *C.humicolus* culture supernatant from incubation supplied with 50 $mg.l^{-1}$ potassium antimony tartrate

	MeSb	Me_2Sb	Me_3Sb
mean supernatant concentration ($ng.ml^{-1}$) (uncorrected)	1.7	30.7	35.3

TRIMETHYLANTIMONY

$$Me_3Sb_{corrected} = 35.3 + \left(35.3 \times \frac{32.7 + 3.5 + 0}{181.8}\right)$$

$Me_3Sb_{corrected} = 42.3\ ng.l^{-1}$

DIMETHYLANTIMONY

Calculation of amount of dimethylantimony arising by demethylation of trimethylantimony species

$$Me_2Sb_{Tri} = \frac{42.3 \times 32.7}{218}$$

$Me_2Sb_{Tri} = 6.35\ ng.l^{-1}$

Calculation of amount of dimethylantimony species present in sample

$Me_2Sb_{Di} = 30.7 - 6.35$

$Me_2Sb_{Di} = 24.35\ ng.l^{-1}$

Correction of dimethylantimony for amount lost by demethylation to lower methylated species

$$Me_2Sb_{corrected} = 24.35 + \frac{24.35 \times (3.5 + 0)}{32.7}$$

$Me_2Sb_{corrected} = 27.0\ ng.l^{-1}$

MONOMETHYLANTIMONY

Calculation of amount of monomethylantimony arising by demethylation of trimethylantimony species

$$MeSb_{Tri} = \frac{42.3 \times 3.5}{218}$$

$MeSb_{Tri} = 0.68\ ng.l^{-1}$

Calculation of amount arising by demethylation of dimethylantimony species

$$MeSb_{Di} = \frac{27.0 \times 3.5}{36.2}$$

$MeSb_{Di} = 2.61\ ng.l^{-1}$

Calculation of amount of monomethylantimony species present in sample

$MeSb_{Mono} = 1.7 - 0.68 - 2.61$

$MeSb_{Mono} = -1.59$

$MeSb_{Mono} = 0\ ng.l^{-1}$

Correction of monomethylantimony for amount lost by demethylation to lower methylated species

$$MeSb_{corrected} = 0 + \left(\frac{0 \times 0}{3.5}\right)$$

$\underline{MeSb_{corrected} = 0\ ng.l^{-1}}$

i.e. the true amounts of mono-, di- and trimethylantimony species detected were 0, 27.0, and 42.3 $ng.ml^{-1}$ respectively.

References for Appendix I

1. M. Dodd, S. L. Grundy, K. J. Reimer, and W. R. Cullen, "Methylated antimony compounds-synthesis, hydride generation properties and implications for aquatic speciation," p. 207. 1992.
2. I. Koch, J. Feldmann, J. Lintschinger, S. V. Serves, W. R. Cullen, and K. J. Reimer, Demethylation of trimethylantimony in aqueous solution during analysis by using hydride generation/gas-chromatography with AAS and ICP-MS detection. *Applied Organometallic Chemistry* **12**, 129 (1998).
3. M. O. Andreae, J.-F. Asmode, P. Foster, and L. Van't dack, Determination of antimony(III), antimony (V), and methylantimony species in natural waters by atomic absorption spectrometry with hydride generation. *Analytical Chemistry* **53**, 1766 (1981).
4. M. O. Andreae and P. N. Froelich, Arsenic, antimony, and germanium biogeochemistry in the Baltic Sea. *Tellus* **36B**, 101 (1984).

APPENDIX II MEDIA RECIPES

Denitrifying medium (Gürleyük *et al.*, 1997) [1]

	$g.l^{-1}$
KNO_3	1.0
KH_2PO_4	7.0
K_2HPO_4	3.0
$(NH_4)2SO_4$	1.0
tri-sodium citrate	0.5
$MgSO_4.7H_2O$	0.1
Glycerol	5 ml

pH adjusted to 7.5

Fecult A medium (Gassmann and Glindemann, 1993) [2]

	$g.l^{-1}$
Bacteriological peptone	5.0
Yeast extract	2.5
Glucose	5.0
KH_2PO4	10.0
$(NH_4)_2Fe(SO_4)_2.6H_2O$	0.4
$CaSO_4.2H_2O$	1.0
$MgSO_4.7H_2O$	2.0

pH adjusted to 5.0

Malt extract broth (Oxoid, Unipath Ltd., Basingstoke, UK)

Nutrient broth (Oxoid)

R medium (Cox and Alexander, 1973) [3]

	$g.l^{-1}$
$(NH_4)_2SO_4$	2.0
Succinic acid	10.9
Sodium succinate	13.5
KH_2PO_4 1%w/v	10.0 ml
$MgSO_4.7H_2O$ 1% w/v	5.0 ml
Thiamine HCl 1%w/v	1.0 ml
after autoclaving:	
Glucose 20%w/v (115psi, 10min)	50.0 ml
$FeSO_4.7H_2O$ 0.36%w/v filter sterilised	0.5 ml

YM medium (Yamada *et al.*, 1996) [4]

	$g.l^{-1}$
Yeast extract	3.0
Malt extract	3.0
Neutralised soya peptone	5.0
Glucose	10.0

Antimony minimal media

	$g.l^{-1}$
$KSb(OH)_6$	2.63
KH_2PO_4	7.0
K_2HPO_4	3.0
$(NH_4)_2SO_4$	1.0
tri-sodium citrate	0.5
$MgSO_4.7H_2O$	0.1
Sodium lactate	2.1 ml

adjusted pH 7.4 by addition of KOH

Antimony-VT mineral medium

	$g.l^{-1}$
KH_2PO_4	0.14
KCl	0.25
$CaCL_2.2H_2O$	0.15
NaCl	1.0
$MgCl_2$	0.62
$NaHCO_3$	1.9
Sodium lactate (70%)	2.1 ml
Vitamin solution	1.0 ml
Trace element solution	1.0 ml
Electron acceptor	5 $mmol.l^{-1}$

adjusted pH 7.4 by addition of KOH

Vitamin solution for basal medium

	$g.l^{-1}$
p-aminobenzoic acid	0.05
Biotin	0.02
Nicotinic acid	0.05
Calcium pantothenate	0.05
Thiamine HCl	0.05
Pyridoxine HCl	0.1
Cyanocobalamin	0.001

Trace element solution for basal medium

	$g.l^{-1}$
HCl	1.0 ml
$MnCl_2.4H_2O$	0.1
$CoCl_2.6H_2O$	0.12
$ZnCl_2$	0.07
H_3BO_3	0.06
$NiCl_2.6H_2O$	0.025
$CuCl_2.2H_2O$	0.015
$Na_2MoO_4.2H_2O$	0.025
$FeCl_3.6H_2O$	1.5

References for Appendix II

1. H. Gürleyük, V. Van Fleet-Stalder, and T. G. Chasteen, Confirmation of the biomethylation of antimony compounds. *Applied Organometallic Chemistry* **11**, 471 (1997).
2. G. Gassmann and D. Glindemann, Phosphane (PH_3) in the biosphere. *Angewandte Chemie International Edition in English* **32**, 761 (1993).
3. D. P. Cox and M. Alexander, Production of trimethylarsine gas from various arsenic compounds by three sewage fungi. *Bulletin of Environmental Contamination & Toxicology* **9**, 84 (1973).
4. R. Yamada, H. Ujiie, Y. Kera, T. Nakase, K. Kitagawa, T. Imasaka, K. Arimoto, N. Takahashi, and Y. Matsumura, Purification and properties of D-aspartate oxidase from *Cryptococcus humicolus UJ1*. *Biochimica et Biophysicts Acta* **1294**, 153 (1996).

APPENDIX III TABULATED DATA AND STATISTICAL ANALYSIS

Due to the low sample population available, a two-tailed student t-test was performed on certain data to ascertain the probability that that the calculated mean and standard deviation deviate from the true values. This statistical analysis was used when two sample populations were compared (and inferences drawn) e.g. does the addition of antimony to *C.humicolus* incubations after 7 days result in a different quantity of involatile methylantimony species in culture supernatants than in incubations where antimony has been present from inoculation (t = 0)?

The two-tailed student t-tests were performed using the free University of Bergen sponsored program available at www.uib.no/isf/people/doc/sttt.htm. Probability values and Degrees of freedom of the relevant assessed data are presented in this Appendix. Data is reported in the text as being significant when there was no statistical difference observed between the means at the 95% confidence level.

Tabulated data from Chapter 2.2

Table AIII.1 Effect of mobile phase molarity upon HPLC separation of trimethylantimony oxide and potassium hexahydroxyantimonate.

Molarity ($mmol.l^{-1}$)	Peak resolution of trimethylantimony oxide and potassium hexahydroxyantimonate
1	1.00 (0.10)
2	1.17 (0.04)
5	0.95 (0.16)
10	0.87 (0.10)
50	0.54 (0.18)

Mobile phase flow rate = 2 $ml.min^{-1}$, pH = 2.8; figure in parentheses are standard deviations based on three replicate injections.

Table AIII.2 Effect of mobile phase flow rate upon HPLC separation of trimethylantimony oxide and potassium hexahydroxyantimonate.

Flow rate ($ml.min^{-1}$)	Peak resolution of trimethylantimony oxide and potassium hexahydroxyantimonate
0.5	1.76 (0.22)
1.0	1.07 (0.09)
1.5	1.01 (0.16)
2.0	0.87 (0.11)
2.5	0.89 (0.10)

Mobile phase molarity = 0.01 $mol.l^{-1}$, pH = 2.8; figure in parentheses are standard deviations based on three replicateinjections.

Table AIII.3 Effect of mobile phase pH upon HPLC separation of trimethylantimony oxide and potassium hexahydroxyantimonate.

pH	Peak resolution of trimethylantimony oxide and potassium hexahydroxyantimonate
2.8	1.76 (0.22)
4.0	0.11 (0.04)
4.5	0.26 (0.04)
5.0	1.61 (0.08)
5.5	trimethylantimony not eluted

Mobile phase molarity = 0.01 $mol.l^{-1}$, flow rate = 0.5 $ml.min^{-1}$; figure in parentheses are standard deviations based on three replicate injections.

Table AIII.4 Effect of acetonitrile content of mobile phase upon HPLC separation of trimethylantimony oxide and potassium hexahydroxyantimonate.

Acetonitrile component of mobile phase (%)	Peak resolution of trimethylantimony oxide and potassium hexahydroxyantimonate
0	0.69 (0.02)
5	1.07 (0.09)
8	0.02 (0.06)
10	0.01 (0.01)
12	-0.10 (0.12)
15	-0.09 (0.08)

Mobile phase molarity = 0.01 $mol.l^{-1}$, pH 2.8, flow rate = 1.0 $ml.min^{-1}$; figure in parentheses are standard deviations based on three replicate injections.

Tabulated data from Chapter 3

Table AIII.5 Total metal analysis of soil and sediment samples by ICP-MS.

Soil/ sediment Sample	Total metal ($\mu g.g^{-1}$ dry weight sample)						
	As	Cd	Cr	Hg	Pb	Sb	Sn
1	17.8 (0.5)	5.0 (0.3)	28.0 (1.4)	1.0 (0.4)	986.0 (49.3)	1.9 (0.1)	6.6 (1.3)
2	2.9 (0.2)	nd	3.1 (0.3)	nd	30.5 (4.1)	nd	0.1 (0.1)
3	2.3 (0.1)	0.3 (0.2)	8.1 (0.2)	nd	106.7 (6.8)	nd	nd
4	2.5 (0.3)	0.3 (0.1)	3.5 (0.2)	nd	53.2 (2.0)	0.7 (0.1)	nd
5	3.1 (0.5)	0.4 (0.1)	20.6 (1.0)	nd	124.0 (9.2)	3.9 (0.4)	nd
6	2.9 (0.1)	nd	5.3 (0.6)	nd	306.0 (15.1)	0.5 (0.4)	nd

Figure in parentheses represents standard deviation of three separate samples, nd = not detected (<0.1 $\mu g.g^{-1}$ dry weight sample). Experimental detail can be found in sections 3.2.1 and 6.2.1. Sample 1 was collected from the watershed (run-off) of a busy road. Sample 2 was collected from a former urban industrial site (not connected with the use of heavy metals). Sample 3 comprised pond sediment taken from a public City park. Sample 4 was collected from a rural field, sample 5 comprised soil sludge associated with vegetation, and was taken from the watershed of a country road, and sample 6 was collected from a garden compost heap.

Tabulated data from Chapter 4

Table AIII.6 Two-tailed t-test of the mean amount of involatile methylantimony species formed during incubation of *C.humicolus* with potassium antimony tartrate ($50mg.l^{-1}$) and the protein synthesis inhibitor cycloheximide. (From Table 4.8).

Treatment	Total amount of methylantimony species after 21 days incubation ($ng.ml^{-1}$)	Degrees of Freedom	Probability value
Sb @ day 0	68.9 (5.1)	-	-
Sb @ day 7	60.8 (5.5)	4	0.1348
CH @ day 3	1.9 (1.5)	4	0.0000
CH @ day 7	49.2 (5.3)	4	0.0097

Additions of potassium antimony tartrate (Sb) and cycloheximide (CH) were made at times shown. Antimony was present from t = 0 in incubations supplied with cycloheximide; figure in parentheses are standard deviations based on three replicate culture incubations; there was no statistical difference between the means at the 95% confidence level when antimony was added at day 0 or day 7, the mean total amounts of methylantimony in incubations with and without cycloheximide were statistically different at the 95% confidence level.

Table AIII.7 Effect of initial substrate concentration (supplied as potassium antimony tartrate) on final supernatant concentrations of methylated antimony species in incubations (19 days) of *C.humicolus*.

Initial substrate concentration ($mg.l^{-1}$)	Supernatant concentration of methylantimony species ($ng.ml^{-1}$)		
	MeSb	Me_2Sb	Me_3Sb
10	nd	nd	9.1 (1.9)
50	2.1 (0.9)	28.8 (4.2)	38.4 (6.4)
100	20.4 (1.6)	72.0 (5.6)	53.6 (6.1)
300	25.4 (3.9)	92.9 (5.8)	65.3 (8.3)
500	13.7 (2.1)	77.0 (2.6)	62.6 (7.6)
1000	1.5 (1.3)	56.6 (3.8)	50.6 (3.1)

Figure in parentheses represents standard deviation of three separate incubations; nd = not detected (< 20 $pg.ml^{-1}$).

Table AIII.8 Effect of initial substrate concentration (supplied as potassium antimony tartrate) on final supernatant concentrations of methylated antimony species in incubations (19 days) of *S.brevicaulis*.

Initial substrate concentration ($mg.l^{-1}$)	Supernatant concentration of methylantimony species ($ng.ml^{-1}$)		
	MeSb	Me_2Sb	Me_3Sb
10	nd	nd	82.3 (3.3)
50	nd	3.1 (1.4)	90,9 (8.2)
100	nd	23.7 (5.1)	175.3 (14.6)
300	16.3 (1.2)	127.5 (5.1)	128.0 (10.6)
500	6.5 (1.2)	52.3 (3.2)	8.3 (0.8)
1000	3.7 (0.8)	49.5 (2.2)	5.4 (3.1)

Figure in parentheses represents standard deviation of three separate incubations; nd = not detected (< 20 $pg.ml^{-1}$).

TableAIII. 9 Two-tailed t-test of the mean amount of involatile methylantimony species formed during incubation of *C.humicolus* with potassium antimony tartrate (50mg.l^{-1}) following incubation with arsenic or antimony as test inducer. (From Table 4.11).

Test inducer	Total amount of involatile methylantimony after 22 days (ng.ml^{-1})	Degrees of Freedom	Probability value
10mg.l^{-1} sodium arsenite	130.4 (5.5)	4	0.0003
50mg.l^{-1} sodium arsenite	182.4 (9.7)	4	0.0001
100mg.l^{-1} sodium arsenite	120.3 (7.1)	4	0.0009
50mg.l^{-1} potassium antimony tartrate	52.1 (6.0)	4	0.2390
No metal	60.8 (9.1)	-	-

***C.humicolus* was incubated with test inducers until mid-stationary phase, at which time test inducers were separated from biomass by centrifugation and washing, and 50 mg.l^{-1} potassium antimony tartrate was added to resuspended biomass as biotransformation substrate; figure in parentheses are standard deviations based on three replicate culture incubations; the total amounts of methylantimony species detected in incubations containing arsenic as test inducer were statistically different from no inducer incubations at the 95% confidence level.**

Table AIII:10 Two-tailed t-test of the mean amount of involatile methylantimony species formed during incubation of *C.humicolus* with potassium antimony tartrate (50mg.l^{-1}) and arsenic. (From Table 4.12).

Co-incubated compound	Total amount of involatile methylantimony after 13 days (ng.ml^{-1})	Degrees of Freedom	Probability value
No arsenic	14.6 (2.2)	-	-
5 mg.l^{-1} sodium arsenite	24.3 (4.7)	4	0.0159
10 mg.l^{-1} sodium arsenite	33.9 (6.2)	4	0.0071
50 mg.l^{-1} sodium arsenite	8.6 (2.1)	4	0.0269
500 mg.l^{-1} sodium arsenite	6.5 (1.5)	4	0.0062
5 mg.l^{-1} sodium arsenate	20.7 (4.0)	4	0.0816
10 mg.l^{-1} sodium arsenate	27.4 (0.5)	4	0.0006
50 mg.l^{-1} sodium arsenate	22.6 (2.5)	4	0.0141
500 mg.l^{-1} sodium arsenate	18.8 (4.4)	4	0.2133

Monomethylantimony species were not detected at any tim; nd = not detected (< 20 pg.ml^{-1}); figure in parentheses are standard deviations based on three replicate culture incubations; the mean amounts of methylantimony species in arsenic containing incubations (excluding incubations containing 5 mg.l^{-1} and 500 mg.l^{-1} sodium arsenate) were statistically different at the 95% confidence level to those omitting arsenic, potassium antimony tartrate was supplied to all incubations at 50 mg.l^{-1}.

Table AIII.11 Two-tailed t-test of the mean amount of involatile methylantimony species detected by HPLC-hydride generation-AFS and hydride generation-GC-AAS analysis methods. (From Table 4.14).

Organism / Antimony substrate	Total methylantimony species detected ($ng.ml^{-1}$)		Degrees of Freedom	Probability value
	HPLC	GC-AAS		
C.humicolus + 50 $mg.l^{-1}$ PAT	67.3 (2.1)	69.3 (3.8)	4	0.4696
C.humicolus + 100 $mg.l^{-1}$ PAT	118.8 (16.8)	146.0 (6.9)	4	0.0604
C.humicolus + 50 $mg.l^{-1}$ ATO	67.6 (3.6)	70.1 (15.1)	4	0.7941
C.humicolus + 50 $mg.l^{-1}$ PHHA	10.0 (2.6)	13.2 (1.9)	4	0.1603
S.brevicaulis + 50 $mg.l^{-1}$ PAT	9.0 (0.8)	11.4 (0.4)	4	0.0097
S.brevicaulis + 100 $mg.l^{-1}$ PAT	131.2 (24.5)	151.7(6.9)	4	0.2355

PAT potassium antimony tartrate, ATO antimony trioxide, PHHA potassium hexahydroxyantimonate. Figure in parentheses are standard deviations based on three replicate culture incubations; there was no statistical difference between the means at the 95% confidence level (excluding *S.brevicaulis* + 50 $mg.l^{-1}$ PAT incubation).

Tabulated data from Chapter 6

Table AIII.12 Profile of lactate and antimony V disappearance, and protein formation in incubations of soil isolate UW-A supplied with potassium antimony tartrate as electron acceptor and sodium lacate as carbon source.

Time (days)	[Lacate] ($mg.ml^{-1}$)	[Protein] ($\mu g.ml^{-1}$)	Antimony V in supernatant ($\mu g.ml^{-1}$)
0	2.89 (0.06)	1.5 (0.5)	1006.2 (20.3)
2	2.38 (0.08)	32.0 (2.7)	912.9 (87.0)
5	1.79 (0.06)	34.0 (3.1)	875.6 (87.3)
7	1.71 (0.04)	35.0 (2.1)	837.6 (38.7)
12	1.59 (0.03)	35.0 (2.3)	802.0 (38.3)
14	1.43 (0.08)	35.0 (3.5)	800.3 (30.9)
19	1.27 (0.07)	35.5 (2.2)	787.6 (79.9)

Figure in parentheses represents standard deviation of three separate incubations; respective concentrations of lacate, protein and antimony V after 19-days incubation of "no biomass incubation"; [lactate] 2.86 (0.03) $mg.ml^{-1}$[protein] 0 (0) $\mu g.ml^{-1}$, [Antimony V] 1000.6 (13.2) $\mu g.ml^{-1}$.